University of Alberta

Biological Factors Affecting Pulp Mill Effluent Induced Coagulation and Flocculation in Receiving Waters

by

Stephanie Anne Joyce

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

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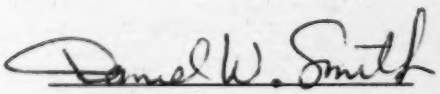

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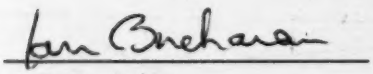

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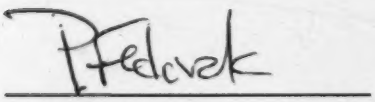

P. Fedorak

ABSTRACT

Recent studies have shown that organic matter discharged from a pulp mill flocculates and accumulates on the river bottom more rapidly than predicted by previous transport models, a phenomenon termed Pulp Mill Effluent Induced Coagulation and Flocculation (PMEICF). PMEICF can cause accumulation of material on the river bottom that may induce anoxic or toxic conditions, subsequently harming benthic organisms and the entire food chain.

From a mixture of effluent and river water, the resulting floc was examined by correlative microscopy, exposing a complex structure. Heterotrophic plate counts revealed a variety of micro-organisms. Individual colonies were isolated, identified and tested for their role in flocculation. Seven isolates were found capable of enhanced flocculation: *Comamonas testosteroni*, species belonging to the *Pseudomonas*, *Enterobacter* and *Aeromonas* genuses and an unidentified isolate. These isolates did induce flocculation, though not consistently. Results varied with the environment available for the micro-organisms. Further tests are required that consider the changing effluent and river water characteristics.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
Al	Aluminium
Alk	Alkaline
BAP	Blood Agar Plate
BOD5	5-day Biological oxygen demand
COM	Conventional optical microscope
DNA	Deoxyribonucleic acid
DOM	Dissolved organic matter
EPS	Extracellular polymeric substances
Fe	Iron
FITC	fluorescein isothiocyanate
GN-ENT	Gram negative-enteric
GN-FAS	Gram negative-fastidious
GN-NENT	Gram negative non-enteric
GP	Gram positive
LC_{50}	Lethal concentration at which 50% of test population dies
MAC	MacConkey solid agar
PME	Pulp mill effluent
PME50	Medium composed of 50% PME
PMEICF	Pulp mill effluent induced coagulation and flocculation
POM	Particulate organic matter
R2A	Prepared solid medium
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RW	River water
SCLM	Scanning confocal laser microscopy
Si	Silicon
SIM	Simulation index
SPAME	Standard plate count agar with 30% PME
TEM	Transmission electron microscope
TSA	Tryptic Soy Agar
TSI	Tripe sugar iron agar
TSS	Total suspended solids
WW	Weldwood

1.0 Introduction

Recent evidence has shown that downstream from a pulp mill effluent discharge, particles form, coagulate and flocculate faster than predicted by current sediment transport models (Krishnappan, 1996b). This phenomenon has been termed pulp mill effluent induced coagulation and flocculation (PMEICF) and is the basis for this study.

Existing sediment transport models have failed to take into account the phenomenon of PMEICF. They assume all particles behave as individual particles and flocculation does not occur (Ongley et al. 1992). PMEICF can cause a build-up of organic material on the river bottom, since insufficient time has passed to allow degradation of the chemicals, if this is possible. Some of the materials may cause adverse conditions, may be toxic or induce anoxic or toxic conditions. The chemicals may still possess a significant biochemical oxygen demand, resulting in low dissolved oxygen in the river. The changed conditions near the river bottom may cause harm to benthic organisms, which could have adverse effects on the entire food chain.

The microbial involvement in biological floc formation is well-documented (Riley, 1963; Pavoni, 1972; Paerl, 1974; Biddanda, 1985; Muschenheim et al., 1989; Rao et al., 1991; Mueller, 1996). Bacteria excrete polymeric substances which may be significant in the floc formation. However, only some bacterial species are considered "floc-formers" (Friedman and Dugan, 1968). Bacteria enter into a starvation-survival phase, when in oligotrophic environments (Humphrey et al., 1983; Kjellberg and Hermansson, 1984), characterized by dwarfing, fragmentation and adsorption to each other or another surface. The physiological changes of the bacteria could influence the observed induced flocculation as well.

This study examined the microbial involvement of the induced floc formation. Through laboratory and some field work, the roles of the bacteria were studied. Attempts were made to determine individual micro-organisms involved and whether or not they alone induce flocculation of the pulp mill effluent.

2.0 Literature Review

2.1 The Nature of Pulp Mill Effluent

2.1.1 Composition

The composition of pulp mill effluent (PME) is extremely variable. It varies among mills, as well as over the year, depending on the type of wood or treatment process being used (Liss and Allen, 1992). Generally pulp mill effluent has an acidic pH and is high in phosphorus and nitrogen (Amblard et al., 1990). When chlorine is added during the bleaching process, chlorinated organics make up a large part of the effluent. These compounds can be referred to as chlorohumus, since the structure is usually unknown (Saski et al., 1994). However, Suntio et al. (1988) published a list of about 250 compounds, most of which are chlorinated, found in a pulp mill effluent. Each one was present at a low concentration but the number of chemicals present is of concern. The major categories of compounds are organic acids and chlorinated organic acids, phenols and chlorinated phenols, chlorinated catechols and guaiacols, sugars, benzene and chlorinated benzene derivatives, aldehydes and chlorinated aldehydes, chlorinated acetone derivatives and chlorinated aliphatics. Structures of some of these are shown in Figure 1. A switch from elemental chlorine (Cl_2) to chlorine dioxide (ClO_2) has greatly decreased the amount of chlorine by-products (Gifford, 1994). Characteristics of these by-products range from water-soluble and rapidly biodegradable substances to persistent and highly bioaccumulative substances such as dioxins and furans (Elliott et al., 1994). This results in various end products and final accumulation sites. PME also contains substantial concentrations of metals, such as zinc, aluminium, copper (Kukkonen, 1996) and in some cases, manganese (AEP, 1997).

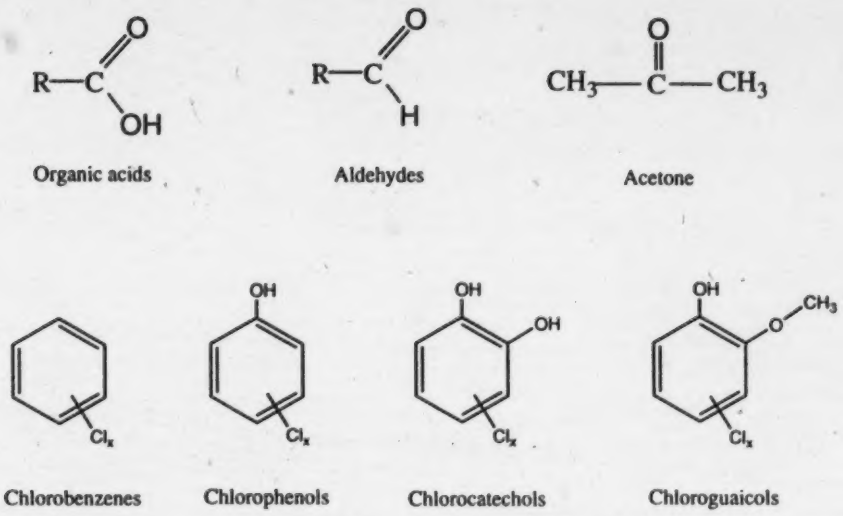

Figure 1: Structures of Some Components of Pulp Mill Effluent.

The R groups can be either alkyl groups or aromatic groups.

2.1.2 Fate of Contaminants

The large volume of wastewater, discharged by pulp mills greatly increases the concentration of dissolved organic carbon of the receiving water. It has a characteristic high concentration of organic halogen compounds because they are relatively resistant to biological treatment (Suntio et al., 1988; Jokela et al., 1993), as well as non-chlorinated organic compounds, such as resin acids (Mohn, 1995). The fate of these compounds is largely dependent on their physical and chemical properties, such as water solubility, water-octanol partition coefficient (K_{ow}), vapour pressure and bioconcentration factor (BCF). In the list of pollutants published by Suntio et al. (1988), some of their physical and chemical properties were published as well. A high water solubility indicates that the compound will likely stay in solution. The K_{ow} estimates the partitioning between the organic and aqueous phases, where octanol is used as a surrogate for organic matter. With a high K_{ow}, the compound is likely to accumulate in an organic phase. The vapour pressure gives a relative volatility of the chemical. The BCF represents the tendency of a chemical to accumulate in aquatic

organisms compared to its concentration in water. All these properties affect the toxicity of chemicals, measured by the LC_{50}. The LC_{50} is the concentration of a particular substance at which 50% of a test population dies. For example, - hexacholorobenzene has a low solubility (0.005 mg/L), a high K_{ow}, a low vapour pressure and a high BCF ($10^{5.46}$ (guppy)). As a result, hexachlorobenzene is extremely toxic, with an LC_{50} of $10^{-2.95}$ mol/m^3 (guppy).

After discharge into the river, the organic components of PME tend to accumulate with organic substances, such as the sediments or biological tissues, or volatilize into the air (Gifford, 1994). The hydrophilic components will likely remain in solution. The intermediates formed during the degradation process may be more biodegradable substances or more persistent compounds (Gifford, 1994). Organisms in the sediment can take up the hydrophobic compounds, initiating accumulation in the food chain (Gifford, 1994). Of concern in this study is that material removed by sedimentation. The organic halogens in particular, have been reported to accumulate downstream of pulp mills (Jokela et al., 1993). Chloroguaiacols and chlorocatechols have been reported to have high sedimentation near the mill, while chlorophenols are not as strongly affected (Kukkonen et al., 1996). The discharge of PME has an effect on the flora and fauna. A drop in algae species diversity and richness, as well as changes in the taxonomic structure of the algal community, have been documented (Amblard et al., 1990). However, an increase in algae production due to the abundance of nutrients has also been documented (Pellinen and Soimasso, 1993).

2.2 Characteristics of Flocculation

Flocculation is an essential process in natural systems because it results in the deposition of fine-grained particles. The settling velocity of flocculated particles can be up to four orders of magnitude greater than unflocculated material as measured from laboratory experiments (Krishnappan, 1996a). Stokes Law, used for cases when the Reynolds number is less than 0.3, cannot be used because of changing particle size. Reynolds number is given in Equation 1, followed by Stokes' law in Equation 2.

$$N_R = \frac{D^2 n \rho}{\mu}$$ **Equation 1: Reynolds' Number**

where D = diameter of the impeller (m)

n = rev/s

ρ = mass density of liquid (kg/m^3)

μ = dynamic viscosity (N·s/m^2)

$$V_c = \frac{g(\rho_s - \rho)d^2}{18\mu}$$ **Equation 2: Stokes' Law**

where V_c = terminal velocity of the particle (m/s)

ρ_s = density of the particle (kg/m^2)

g = acceleration due to gravity (m/s^2)

d = diameter of particle (m)

The occurrence of flocculation corresponds to type 2 versus type 1 settling. Type 1 settling is discrete particle settling, without significant interaction between neighbouring particles. Type 2 settling involves coagulation and flocculation, resulting in an increased mass of the particle and increasing settling velocity (Tchobanoglous and Burton, 1991). For flocculation to occur, two conditions must be fulfilled: 1) a collision between particles and 2) adhesion between particles (van Leussen, 1988). The collision frequency is increased with increasing turbulence, but in highly turbulent waters the floc particles may shear apart. Thus a maximum floc size is obtained.

Factors affecting the collision stage of flocculation include velocity gradients within the suspending liquid (important for particles >10 μm), differential settling of particles (>10 μm) and Brownian motion (<1.0 μm) (Krishnappan, 1996a). Collisions caused by Brownian motion are called perikinetic flocculation and those caused by velocity gradients are termed orthokinetic flocculation (van Leussen, 1988). Perikinetic flocculation tends to result in ragged, weak flocs. Orthokinetic flocculation tends to form

spherical flocs that are stronger than those formed by the process. Differential settling results in ragged weak flocs, but is most efficient at clearing water.

There are four main cohesion mechanisms (van Leussen, 1988; Krishnappan, 1996a). The first, salt flocculation, is dominant when there is a high concentration of cations. In solutions of high ionic strength, the double layer surrounding each particle is compressed, facilitating aggregation. In river systems, this process is not as important as the other three (Droppo and Ongley, 1992). The second mechanism is the formation of organic aggregates and bioflocculation. Following colonization of inorganic particles by bacteria, polysaccharides are produced by a process in which dissolved organic matter (DOM) is converted to particulate organic matter (POM). The polysaccharides adsorb onto other inorganic particles, enhancing flocculation by inter-particle bridging. Further adsorption and colonization of bacteria continue this process. The third mechanism is pelletization. Filter feeders, such as copepods, transform suspended matter into pellets, which have faster settling velocities. The final mechanism involves the chemical coatings of particles. These control the charge of the particle that will affect its cohesive abilities.

The properties of the floc particle that are affected by its environment are its size, density and strength. Growth of the floc particle has been characterized by distinct structures. At the lowest level, the inorganic particles, held together with uniform porosity are considered zero-ordered aggregates. Several of these particles together are called first-order aggregates. Several first-order aggregates clumped together form a second-order aggregate and so on. Most experimental evidence reveal a 3- or 4-level floc structure (van Leussen, 1988). As the order increases, the diameter of the floc will increase, as well as its porosity. This decreases the density as well as the floc shear strength (van Leussen, 1988; Droppo et. al., 1997). It is important to remember that floc size is a dynamic property, depending on the rate of aggregation, the rate of break-up (determined by the turbulence) and other environmental factors. A summary of the factors affecting floc size and structure is illustrated in Figure 2.

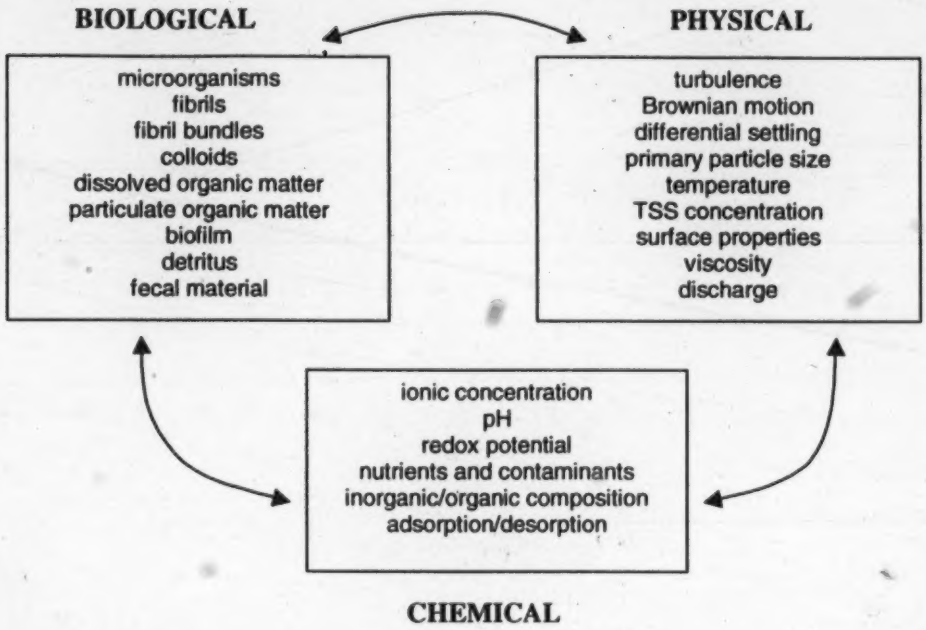

Figure 2: Factors Affecting a Floc Particle (adapted from Droppo et al., 1997)

The aggregated material found in rivers may be of three forms: 1) water-stable soil aggregates washed into the system via overland flow, 2) sediment flocculated within the river by physical, chemical and biological means and 3) a combination of the above (Droppo and Ongley, 1994). The formed particles consist of micro-organisms and silt particles (van Leussen, 1988). The size of the floc can range from a few microns to several millimetres (van Leussen, 1988).

Much of the past research has focussed in natural flocculation in estuaries (Riley, 1963; van Leussen, 1988; Muschenheim et al., 1989; Eisma et al., 1991). This differs greatly from river flocculation, due to the high ionic strength of the marine waters. More recently, flocculation in rivers has gained in interest (Krishnappan, 1996a). River flocs tend to be smaller in size, due to the increased turbulence and low cation concentration (Droppo and Ongley, 1992). The lower ionic strength increases the energy barrier (i.e. the repulsive forces between the particles), inhibiting flocculation (Droppo and Ongley, 1994), as illustrated in Figure 3.

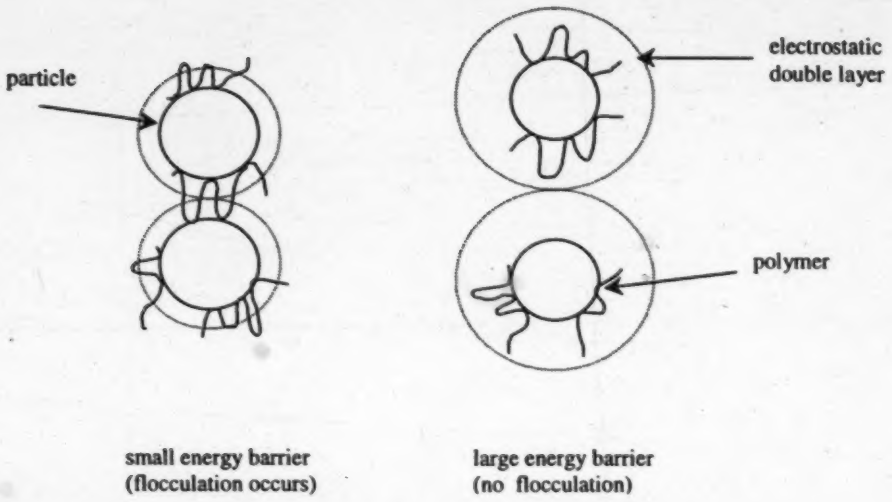

Figure 3: Bridging of the Energy Barrier by Polymers (adapted from van Leussen, 1988)

2.3 Microbial Involvement

2.3.1 Extracellular polymeric substances

Bacteria can be surrounded by a layer of organic material. This may be in the form of a capsule or slime layer. A capsule consists of organic matter that adheres to the cell wall, whereas a slime layer extends into the suspending medium, with finger-like appendages (Geesey, 1982). This organic layer is often referred to as extracellular polymeric substances (EPS). Most are composed of polysaccharides, but some can be proteinaceous (White, 1995) or strands of RNA or DNA (van Leussen, 1988; Pavoni et al., 1972). The exact chemical composition varies with the type of micro-organism and the environmental conditions (Geesey, 1982). They have a high length-to-width ratio, with a diameter ranging from 2 to 20 nm (Leppard, 1986).

EPS play an important role in the life of a bacterium. They anchor the bacterium to a protective surface, to the food source, or to other micro-organisms (Geesey, 1982). Being attached may offer ecological advantages (Flemming, 1993). Bacteria are less exposed to solar radiation and predator-prey interactions are reduced in this condition (Harvey and Young, 1980). EPS also provides cohesion and structural

integrity to the micro-organism (Flemming, 1993). Other research has shown that EPS can interact with and transport pollutants (Leppard, 1986), can complex and detoxify metal ions (Geesey, 1982; Leppard, 1986) as well as transport enzymes (Leppard 1986). EPS also aid bacteria in flocculation, but they are not necessary for flocculation to occur (Friedman and Dugan, 1968).

2.3.2 Colonization and the Formation of Aggregates

EPS play an essential role in the colonization of sediments by bacteria. They aid in the attachment of bacteria to solids (Geesey, 1982; Biddanda, 1985). Not all bacteria attach to sediments. However, studies show that the number of free bacteria in a solution decreases with time, indicating that more and more bacteria have attached to the sediment (Rao et al., 1991) or died off. The concentration of attached bacteria is usually greater than the concentration of free bacteria (Goulder, 1977).

Microscope work has shown that flocs viewed under high resolution resemble microbial biofilms, described in the literature (Liss et al., 1996). The colonization and growth of a biofilm at a solid-liquid interface has been described by Mueller (1996). In this case, the sediment particles can be considered the solid material. The process is broken down into six steps: 1) substratum conditioning by organic molecules, 2) transport of cells to the surface, 3) adsorption of cells to the substratum, 4) transformation of reversibly adsorbed cells to irreversibly adsorbed cells, 5) growth of the biofilm and 6) erosion of cells. The third step has been subsequently studied. The bacteria tend to colonize on a surface in a planar manner, until a critical size of the biofilm is reached. Further growth is away from the surface, creating a complex 3-D biofilm (Allison and Sutherland, 1987). The fifth step, growth of the biofilm, can lead to growth of the aggregate. This has also been subsequently studied (Muschenheim et al., 1989). An immediate aggregation of particles was observed, due only to interparticle collision (i.e. a physical process). The time scale was too short to include any biological processes. A second biologically-mediated stage occurs, that results in larger aggregates. This stage involved the attachment by bacterial exudates (Biddanda, 1985; Muschenheim et al., 1989). In laboratory studies, this delayed flocculation was

shown to correspond to the time when the micro-organisms enter into an endogenous growth phase (Pavoni et al., 1972).

Upon attachment to the sediment, polymers extend out into the surrounding solution, where they may attach to another particle. This mechanism of aggregation is termed interparticle bridging. There are considered to be a finite number of adsorption sites on a particle. If too few sites are occupied by polymers, bridging may be weakened and the floc is easily broken. If too many of the sites are covered (by particles other than polymers) then the number of free sites available for polymers is limited and flocculation is hindered (van Leussen, 1988). Thus an optimum amount of polymer exists.

For bioflocculation to occur, the polymers must bridge the electrostatic double layers of both particles (see Figure 3, in section 2.2). The presence of some cations in the solution will compress the double layers and promote flocculation (van Leussen, 1988). Studies have shown that the presence of cations is essential for flocculation (Busch and Stumm, 1968; Levy et al., 1992; Sanin and Vesilind, 1996).

Bacteria have been shown to mediate the transfer of DOC to particulate organic carbon. This contributes to the larger aggregate size (Riley, 1963; Paerl, 1974). Aggregation is affected by seasonal variation. Riley (1963) reported that the quantity of aggregate material peaks in winter, followed by a rapid decrease until April. It then increases into the early summer months of May and June and decreases in July. Goulder (1977) showed that attached bacteria were more numerous in winter months than warmer months.

2.3.3 Starvation of Bacteria

In oligotrophic environments (environments where there are few nutrients), copiotrophs (micro-organisms requiring large amounts of nutrients) enter into a starvation-survival phase (Humphrey et al., 1983). Generally this consists of two steps: 1) dwarfing and 2) fragmentation. Dwarfing is simply a reduction in size and fragmentation results in an increase in individual cell number, but without growth (Mueller, 1996). Starved bacteria show an increase in swimming speed and a slight reduction in the length of random free runs. They appear to have a greater affinity for

adhesion, a possible survival mechanism (Mueller, 1996). Much variation exists among bacterial strains in their response to low nutrient conditions (Kjelleberg and Hermansson, 1984). The length of time from the onset of starvation for dwarfing and fragmentation to occur varied greatly.

The importance of this starvation phase is that bacteria are considered "stickier" and will attach to organic matter more readily. Mueller (1996) suggested that this attachment could be used as a survival mechanism. Pavoni et al. (1972) concluded that bacterial bioflocculation would not occur until the bacteria are in an endogenous growth phase.

2.3.4 Bacterial Species

Some research has been done into the particular bacterial species that are involved in floc formation. In general, bacteria can be classified as floc-forming or non-floc-forming (Friedman and Dugan, 1968). Floc-forming bacteria tend to clump together when in liquid suspension. This is not always associated with the production of a capsule or slime layer (Friedman and Dugan, 1968). Most studies have attempted to isolate floc-forming bacteria from activated sludge. McKinney and Horwod (1952) identified 12 floc-forming organisms. Five were identified as *Bacillus cereus*, *Escherichia intermedium*, *Paracolobactrum aerogenoides*, *Nocardia actinomarpha* and a *Flavobacterium* spp. Friedman and Dugan (1968) added *Zoogloea ramigera* to this list. A study by Dudley et al. (1980) focussed on human pathogens and identified other species in both sludge and sewage. Al-Shahwani et al. (1986) showed that floc formation was enhanced when *Klebsiella* spp., *Escherochia coli* and *Streptococcus* spp. were present. Work by Roth et al. (1989) showed that aggregates of *Zoogloea* spp. were present during early floc formation. However limited work has been done to identify floc-forming bacteria from pulp mill effluent.

The other general focus of research in this area had involved identifying bacterial species in pulp mill effluent, responsible for the numerous reactions occurring. Recently, Fulthorpe et al. (1993) identified and characterized a large number of the culturable bacteria in pulp mill effluent. There is general consensus amongst microbiologists that the majority of bacteria in complex natural communities cannot

form colonies on the traditional media used in laboratories (González et at., 1996). Tsernoglou and Anthony (1971) showed that direct counting methods revealed a much larger microfauna than culture methods. The isolates belonged to phenetic clusters, that were identified as *Acinetobacter* spp., *Acidovorax* spp., *Pseudomonas* spp., *Ancyclobacter aquaticus*, *Klebsiella* spp. and an unidentified cluster of pleomorphic, Gram negative methylotrophs. The first three listed were common in the river water, while the latter two were common in the mill treatment system. The majority of the isolates did not match the known fingerprints in the Biolog GN database, which contains >500 species (Fulthorpe et al., 1993). Some strains were reported to be present in both the river water and the mill treatment system. These were of the *Pseudomonas* group, including *P. stutzeri*. *A. aquaticus* was the largest of the clusters and was isolated on medium containing some clarifier effluent. The unidentified cluster, named cluster C by Fulthorpe et al. (1993), was composed of deep yellow or pale orange pigmented bacteria. They were oxidase and catalase positive, pleomorphic rods of variable thickness, or L- or C-shaped cells. *A. aquaticus* and the unidentified group were able to dechlorinate simple chlorinated aliphatics.

One bacterial species, mentioned by Geesey (1982) produces an "elaborated sheath" around the bacterial cell wall. *Sphaerotilus natans* has been found in pulp mill effluent and mine drainages (Geesey, 1982). This sheath could play a role in flocculation.

Bacteria with specific degradation abilities have been isolated as well. Mohn (1995) isolated bacterial species from a sequencing batch reactor in a paper mill. The bacterial species were able to grow on the resin acid dehydroabietic acid. Two of these isolates were found to be most closely related to *Sphingomonas yanoikuyae* and *Zoogloea ramigera* (by analysis of the small subunit rRNA partial sequence). Attempts have been made to identify bacteria involved in lignin degradation from a marine pulp mill enrichment culture (González et al., 1996). The majority (8 of 14) of these bacteria are in the α-subclass of *Proteobacteria* with one in the γ-subclass, three in the *Cytophagia-Flavobacterium* group and two were Gram positive. González et al. (1997) isolated two new species from a marine, lignin-rich pulp mill effluent. The species proposed were *Microbulbifer hydrolyticus* and *Marinobacterium georgiense*.

M. hydrolyticus was able to break down cellulose, xylan, chitin and gelatin and *M. georgiense* utilized mono- and disaccharides, alcohols, amino acids, methanol and aromatic compounds for growth. Thus it is evident that a wide variety of bacterial species exist in pulp mill effluent, the majority of which have not been identified.

Liss and Allen (1992) investigated the changing microbial community in a pulp mill aerated lagoon, over a 12-month period. The total bacterial count remained fairly constant throughout the year, however the numbers of aerobes, anaerobes and psychrotrophs did fluctuate. No attempt at identification was made.

2.4 The Role of Pulp Mill Effluent and the River System in Flocculation

Much uncertainty surrounds the ability of pulp mill effluent to induce floc formation in rivers. Initial field studies by Krishnappan (1996b) revealed that the particle size distribution downstream of the pulp mill effluent discharge at Hinton, Alberta was significantly different than the upstream particle size distribution. This was measured in-situ, using a new submersible laser particle size analyzer. Downstream of the discharge, the concentration of every size of particle had decreased, implying that the particles had settled, and the settling had been a result of flocculation.

In a subsequent laboratory study, Krishnappan (1996b) demonstrated this, using pulp mill effluent from the Weldwood of Canada Ltd. pulp mill in Hinton, Alberta, and a rotating flume. The effluent was introduced into the flume at a concentration similar to what is found in the river, during low flow conditions. The deposition rate was found to be higher than for the control, which had no effluent introduced. As well, the sediment flocs were larger in the test situation. This was the first evidence of PMEICF.

This research sparked another study with effluent from the Northwood Pulp Mill on the Fraser River in Prince George, British Columbia (Evans, 1996). These results showed that aggregation of the particles within the flume may occur, but the effect was slight and would not affect the overall transport of sediment in the Fraser River.

Additional confirmatory studies (Yang and Smith, 1999) using effluent from both the previously mentioned Weldwood site and the Weyerhaeuser pulp mill in Grande

Prairie have shown that PMEICF does occur. What remains to be known is whether this is a physical-chemical process or a biological one, and whether the effect is significant in the river systems studied.

2.5 *Biolog Identification System*

The Biolog System was chosen for use in this study because it involves a minimum number of biochemical tests to identify the bacteria species. Before initiating the Biolog procedure, the bacterial isolates need to be classified as Gram positive (GP), Gram negative, enteric (GN-ENT), Gram negative, non-enteric (GN-NENT) or Gram negative, fastidious (GN-FAS). The numerous Gram negative classifications (GN-ENT, GN-NENT, GN-FAS) reflect that fact that PME is mainly composed of Gram negative bacteria (Fulthorpe et al., 1993; Liss et al., 1996) and only the Gram negative software was available for use at the time of testing. The tests required for classification into the one of the above categories are the Gram stain, oxidase and catalase, reaction in a triple sugar iron (TSI) slant and growth on a MacConkey (MAC) plate. The classifications are shown in Table 1.

Table 1: Classification of Bacteria from Biochemical Tests

Classification	Gram	MAC	TSI	Oxidase
GP	Positive	No growth	-	-
GN-ENT	Negative	Growth	Acid or Alk/Acid	Negative
GN-NENT	Negative	Variable	Alk/Alk	Variable
GN-FAS	Negative	No growth	No growth	Variable

The GN2 MicroPlate™ contains 96 wells, 95 of which are filled with different carbon sources, and 1 control well. A map of the 96 wells can be found in Appendix C. All required nutrients and biochemicals are added to the 96 wells and subsequently dried. In addition, the redox dye, tetrazolium violet, is added to each well. The isolate to be identified is suspended in a special inoculating fluid (GN/GP-IF, provided by Biolog Inc.), at a specified concentration. Sodium thioglycolate (Figure 4) is added to the cell

suspension of GN-ENT and GN-FAS bacteria. It is an anti-capsule agent that inhibits the colour change in the control well and negative wells that could occur when the bacteria use their polysaccharide capsules as a carbon source. Exactly 150 µL of the cell suspension is then injected into each of the 96 wells and the MicroPlate is incubated for the specified length of time.

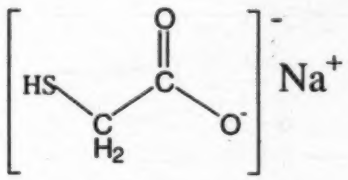

Figure 4: Structure of Sodium Thioglycolate ($C_2H_3NaO_2S$)

During the incubation period, if the bacterial isolate is able to utilize the carbon source, the carbon source is oxidized, indicating that respiration is occurring. This causes the redox dye to change from colourless to violet. Its structure is shown in Figure 5.

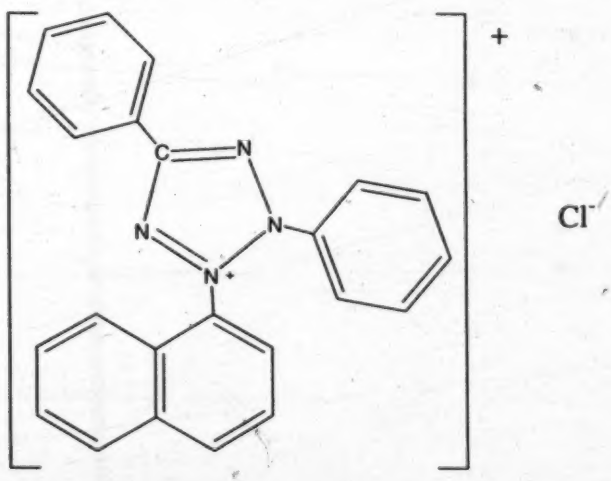

Figure 5: Structure of Tetrazolium Violet ($C_{23}H_{17}ClN_4$)

At the end of the incubation period, the pattern of purple and clear wells is recorded, which is referred to as the fingerprint. The fingerprint is entered into Biolog's Microlog computer program and if the simulation index (SIM) is adequate, the identification is made. A SIM of 0.75 is required for plates read after 4 to 6 h of

incubation and a SIM of 0.5 is required for those read after 16 to 24 h. Whether or not an identification is made, the computer program will list the top ten potential IDs, with the associated probability and SIM. Thus, if no identification is made, possible IDs are listed and the top one may still be the correct name of the micro-organism present. Using the SIM, it can be deduced whether or not it is a valid assumption. For example, a SIM of 0.49 for a non-enteric bacteria species would have a greater possibility that the corresponding name is correct, than if the SIM was 0.39.

In the past, research showed that the Biolog identification system has not been very successful at identifying environmental bacteria. Fulthorpe et al. (1993) used the Biolog GN-MicroPlate™ and found that the majority of their isolates from PME did not match any of the known fingerprints. The Biolog fingerprints, as well as further taxonomic tests were used to identify the bacteria. González et al. (1996) used Biolog in addition to two whole genome approaches to identify their isolates from PME. However, the Biolog GN-MicroPlates™ were used solely for biochemical characterization. This revealed any duplicate isolates that kept DNA sequencing to a minimum.

A comparison of phenotypic and genotypic techniques for the identification of Gram negative pathogenic bacteria was performed (Tang et al., 1998). Phenotypic techniques rely on traits that are expressed by the organism, such as carbon source utilization and biochemical pathways. Identification based on carbon source utilization is fundamental to the Biolog system. The Sherlock system (MIDI, Inc.) is based on cellular fatty acid profiles, obtained by gas-liquid chromatography. These are then compared to a database, similar to the Biolog system. Problems with these phenotypic techniques arise because individual tests might not be reproducible and the expressed phenotype may not be absolute and vary among species (Tang et al., 1998). Genotypic identification involves the sequencing of a part of the genetic material of the organism. The method used by Tang et al. (1998) identified bacteria based on the sequence of their 16S rRNA gene. The 16S rRNA refers to a strand of RNA associated with one of the subunits of the ribosome. The associated gene would code for this information. Thus it is simply a strand of genetic material (Tortora et al., 1992; White, 1995). This particular gene is used because it is highly conserved during evolution. In this comparison study, 63 of 72 isolates were identified to the genus level by the Biolog system, compared to 70 of 72 by the 16S

rRNA gene sequencing method. Ten of these could not be identified at the species level based on phenotypic techniques alone. Thus the Biolog system seems to be more successful for pathogenic bacteria than environmental bacteria.

2.6 Correlative Microscopy

Correlative microscopy is a technique that involves examining the specimen under more than one microscope, to obtain a more vivid image of the 3-D structures. The microscopes that are generally used are the conventional optical microscope (COM), the transmission electron microscope (TEM) and the scanning confocal laser microscope (SCLM). Given that the nature of floc particles is highly unstable and fragile, a stabilization technique has been developed that permits observation of the same sample under all three microscopes. This was developed by Droppo et al. (1996a and 1996b).

A floc sample is allowed to settle in a column standing on a glass slide. Following settling, the column is removed, leaving the floc in a small amount of water on the slide. The floc can be observed directly like this, or the remaining water can be removed and an agar solution added. This solidifies in 1 to 2 min, leaving a round agar disk that can be transported without breaking the floc structure. It can be cut to allow examination of the same sample by all three microscopes.

The COM shows the entire floc structure, up to a 40-fold magnification. From this, the size, shape and volume can be estimated. These properties are important for modelling sediment transport and settling (Liss et al., 1996). The TEM produces an image of the fine structured details in the floc. Any bacterial coating and/or EPS substances present may be visible with the TEM (Leppard, 1992). The SCLM can be considered a bridge between the COM and TEM. It is able to produce an image of cross-sections, 0.5 µm apart and then assemble the images to form a 3-D picture.

Work by Liss et al. (1996) and Droppo et al. (1997) used correlative microscopy to study the freshwater floc. A complex internal structure was revealed: a mixture of organic and inorganic components, held together by a fibrillar matrix. Bacteria, present within the floc matrix, secrete the fibrillar material. The authors believed that these polymeric fibrils are the dominant material involved in the development and stabilization

of the floc particle. The presence of pores within the floc structure was also evident. The pores appeared to be void, but under higher resolution, they were found to be filled with a 3-D matrix of polymeric fibrils. Liss et al. (1996) also studied the engineered floc; a floc particle isolated from a pulp and paper mill oxygenated activated sludge effluent system. The TEM revealed that bacteria are more abundant in the engineered floc particles, than the freshwater ones. Both bacteria-bacteria associations and EPS were present. The inorganic material present in the engineering floc was similar to the inorganic material found in the freshwater floc (Fe, Si and Si substituted with Al, determined by energy dispersive spectroscopy), although it was more abundant in the freshwater floc.

3.0 Materials and Methods

3.1 Preliminary SEM Studies

Initial studies involved examination of the floc for the presence of bacteria. A 1:1 mixture of PME and RW from the Weyerhaeuser site was left mixing for up to 52 h. At 4.5, 28 and 52 h, 9 mL was taken from the sample and added to 6 mL of a 2.5% gluteraldehyde solution. This stood for about 1 h before filtration through a 0.2 μm membrane filter (Gelman Sciences). The gluteraldehyde solution is a fixative to preserve the structure of the bacteria. Simultaneously, samples of the PME alone and the RW alone were taken and treated the same way (with no mixing time). All samples were then prepared for the SEM following the standard procedure. The sequence of steps taken were ethanol drying, critical point drying, mounting, sputtering with gold and finally observation by SEM. The presence and appearance of the bacteria were noted. The SEM used was a HITACHI S-2500 SEM, located in room 1148 of the Dentistry-Pharmacy building on the University of Alberta campus.

On the Athabasca River in Hinton, samples were taken at various locations upstream and downstream of the discharge, and examined by SEM. They were collected in 200 mL polystyrene bottles, with 200 mg of sodium thiosulphate (Fisher) added, to preserve the bacteria. An upstream sample was taken, as well as samples at approximately 100 m and 2 km downstream. The same preparation procedure for the SEM was used as mentioned previously. Again, the presence and appearance of the bacteria were noted.

3.2 Heterotrophic Cultures

Heterotrophic plates were performed on samples from the PME, the RW and a mixture of the two. Samples were collected from two sites: Weldwood of Canada Ltd. PME with Athabasca RW and Weyerhaeuser Canada PME with Wapiti RW at the end of August and beginning of December 1998. They were collected in unsterilized 20 L buckets. It was assumed that that PME and RW bacteria would be more abundant than any contamination and by repetition of the experiments, the regularly-occurring species

would be from the PME or RW. Samples were shipped by Purolator, which took about 2 d. They were stored in the cold room at 4°C, until needed (up to 4 weeks). Using a jar test apparatus, 2 L of the final effluent was placed in one jar, 2 L of the corresponding upstream river water in a second and 1 L of effluent and 1 L of river water in a third (see Figure 6) and covered with tin foil to prevent contamination from airborne microbes. The jar test apparatus was not sterilized before use, since the jars could not be autoclaved and washing with Presept (500 mg/L sodium dichloroisocyanurate, Fisher) did not completely sterilize them. Thus, it was assumed that the bacteria present in the PME and RW would be more abundant than any contamination. The jar test apparatus was left mixing for up to 4 d at a speed of 10 rpm. Samples were taken from both the liquid phase and the floc material that formed in the jar. Liquid samples were taken after 1 d and after 4 d by a sterile 10 mL pipet. Floc samples were only taken after 4 days to allow for attachment and establishment of any bacteria. For these samples, the floc was allowed to settle (i.e. the paddles were turned off). Once it had settled, the floc was sampled by a sterile 10 mL pipet into a small beaker. This was allowed to settle and the liquid was decanted off the top. A small amount of dilution buffer (0.1% (w/v) Bacto™Peptone, Difco) was added and the mixture shaken. This mixture was diluted numerous times to obtain isolated colonies on at least one plate. Six serial 10-fold dilutions were used for both the floc and the liquid samples. Each was shaken briefly before the next dilution was performed. All samples were filtered through a 0.45 μm black membrane filter (Gelman Sciences). Three types of media were used: 1) R2A (Difco), 2) a mixture of standard plate count agar (Difco) with 30% (v/v) PME (abbreviated SPAME) and 3) a medium composed of 50% by volume PME, 50% by volume deionized distilled water and 2 % (w/v) agar (abbreviated PME50). Nutrients present in the prepared media are given in Appendix A. Plates were incubated for up to 5 d, to allow for growth of slow-growing organisms. The bacterial colonies present on the plate, after 48 hours of growth, were evaluated qualitatively. The colour, texture and nature of the edge of the colonies were noted. Experiments were repeated until it was evident which colonies were abundant in the sample, based on appearance only. These colonies were isolated.

Figure 6: Jar Test Apparatus

Present in the jars, from left to right: 2 L Weyerhaeuser (WH) PME; 1 L WH PME and 1 L Wapiti RW; 2 L Wapiti RW; 2 L Weldwood (WW) PME; 1 L WW PME and 1L Athabasca RW; 2 L Athabasca RW.

3.3 Bacterial Isolation and Identification

From the culture plates obtained in section 3.2, individual colonies were isolated by streak plating onto the same medium from which they were obtained (R2A, SPAME or PME50). Isolates were streaked onto solid medium a minimum of 5 times, to ensure purity. They were then grown in a similar liquid broth and kept frozen in a glycerol solution at -70°C until needed. To resume growth, bacteria were thawed and a small amount of freezing solution was transferred to a test tube containing the corresponding broth. The test tubes were incubated until growth was visible (turbid), from 1 to 3 d. From the test tubes bacteria were sub-cultured onto blood agar plates (BAP), MacConkey (MAC) plates and the original medium from which they were obtained. These were incubated overnight (or longer, if needed). The Gram stain and catalase and oxidase tests were performed as well as inoculation of a triple sugar iron (TSI) slant. Procedures for these tests are given in Appendix B. The results of all of these tests enabled classification of the bacteria into a Gram positive (GP), Gram-negative enteric (GN-ENT), Gram negative non-enteric (GN-NENT) or Gram negative fastidious (GN-FAS) category (see Table 1 in section 2.5). These categories specify testing conditions for the Biolog (Table 2). They were also classified as good, poor ⊕ or poor growers, based on their growth behaviour on BAP or R2A. Poor ⊕ is a middle category, for isolates that exhibited growth behaviour between good and poor growers.

Table 2: Testing Conditions Specified by Biolog Inc.

Parameter	GN-ENT	GN-NENT	GN-FAS
Subculture time	16 to 24 h	16 to 24 h	24 to 48 h
Subculture medium	TSA with blood	TSA with blood	Chocolate
Subculture conditions	35°C	30°C	35°C/CO_2
Inoculum concentration	63% (turbidity reading)	52% (turbidity reading)	20% (turbidity reading)
Supplement	Thioglycolate	NA	Thioglycolate
Incubation conditions	35°C/humidity	30°C	35°C/CO_2
Incubation time	4 to 6 h	16 to 24 h	16 to 24 h
SIM for ID	0.75	0.5	0.5

The MicroPlates were inoculated following the standard procedure outlined by Biolog Inc. The layout of the GN2-MicroPlate™ is shown in Appendix C. They were incubated for the specified amount of time and the "fingerprint", the pattern of purple and clear wells, was recorded. A purple well indicated a positive reaction (+) and a clear well indicated a negative reaction (-). If the well was faintly purple or only specs of purple were visible then this was indicated as borderline (/). This was entered into the computer program, which returned a top ten list of possible IDs, for the isolate.

3.4 Re-Introduction of Isolates into Sterile Effluent

Before any re-introduction of the isolates, the effluent required sterilization. Since the physical and chemical properties of the effluent needed to remain unchanged, two methods were chosen and evaluated. The methods were autoclaving the effluent and adding chloroform. Autoclaving the effluent involved three separate treatments, over 4 d. This ensured that any spore formers that could withstand one treatment, would be killed by the third treatment. Each treatment was at 120°C for 30 min. The PME and RW samples were autoclaved in 1 L Erlenmeyer flasks, covered with tin foil. As a second method of sterilization, chloroform was added at a concentration of 0.5% (v/v). The chloroform was volatile, so more had to be added each day. Heterotrophic plates were

used to test for sterility and the turbidity and the particle size distribution were measured and compared to the straight effluent.

Approximately 11 mL of each pure culture (prepared growth medium and bacterial growth), isolated and identified in section 3.3, was introduced into a mixture of 900 mL of sterile effluent and 900 mL of sterile RW, to determine if the bacteria could induce flocculation. A sterile, 2 L Erlenmeyer flask was used, with mixing provided by a stir bar, as shown in Figure 7. The mixing environment was kept as constant as possible. A variety of stir plates and stir bars were used, so the mixing speed was determined by the size of the swirl present at the surface of the mixture. Three controls were used: 1) 900 mL of sterile effluent and 900 mL of sterile river water with no inoculum added, 2) 900 mL of untreated effluent (with its natural fauna) and 900 mL of river water and 3) 900 mL of sterile effluent and 900 mL of sterile river water, inoculated with 11 mL of untreated effluent. The turbidity and particle size distribution were used to measure the extent of flocculation. An initial reading (turbidity and particle size distribution) immediately following set-up was taken, as well as a final reading 4 d later. Any sampling for these measurements between these times risked contaminating the sample. For the turbidity reading, 20 mL of the mixture was placed in the small turbidity jar. The turbidity was read in an Orbico-Helling Digital Direct-Reading Turbidimeter. For the particle size distribution measurements, the mixture was diluted in a 1:100 ratio, in a 500 mL flask (i.e. 5 mL of sample). The particle size distribution of this mixture was read from a HIAC/ROYCO 8000 particle size analyzer, which included the dilution factor in its results.

Figure 7: Erlenmeyer Flask Used for Flocculation Experiments

In the flask is 1 L of Weyerhaeuser PME, 1L of Wapiti RW, 11 mL of bacterial culture (HJ) and a stir bar, visible at the bottom. The photo is taken after 4 d of stirring.

As a validation procedure, samples from the mixtures were re-plated, to determine if only the one re-introduced species was present. The sterile control was tested for sterility. Samples were taken from only the liquid phase, diluted and plated onto the corresponding media (R2A or SPAME). If only one species was present on the plate(s), and its colonial morphology resembled that of the inoculant, it was assumed that the mixture was pure and that species was responsible for any flocculation. If two or more species were visible (i.e. an impure solution), the experiment was repeated. For the first experiments this occurred quite often. However, as the procedure became familiar, this occurred about once every 10 experiments.

3.5 Observation of Floc Structure

A 1:1 mixture of PME and river water were mixed for 4 d in an Erlenmeyer flask such as those used in section 3.4. A stir bar provided adequate mixing, without allowing the floc to settle. A sample of the mixture was placed into the cylinder of the apparatus shown in Figure 8. The floc was allowed to settle for 24 h, the liquid was removed by pipet, and then the cylinder was removed, leaving the floc on the glass slide. A 0.75% (w/v) agarose solution was added to stabilize the floc as an agarose disk without disrupting the appearance of the floc. Once the agar had solidified, the disk was stained with fluorescein isothiocyanate (2 mg/mL of 10 mM Tris-HCl (pH 10.0), Sigma). The disk was then washed a minimum of three times with 0.1 M phosphate buffered saline (pH 7.0). The agarose disk was placed on a sterile petri dish, halved and examined under three different microscopes: COM, TEM and SCLM. This method was obtained from Droppo et al. (1996a and b). All three microscopes were used in room CW225 of the Biological Sciences Centre on the University of Alberta campus.

Figure 8: Apparatus Used for Floc Settling

4.0 Results and Discussion

4.1 Preliminary SEM Studies

For the preliminary SEM studies involving the timed experiments and the PME and RW photographs, the samples were taken from the Weyerhaeuser site in June 1998. Unfortunately, no characteristics of the RW were obtained at this time. As it was spring when the sampling was done, the river likely had a high sediment load at this time. As for the PME, the TSS had a concentration of 11 mg/L and was discharged at a rate of 559 kg/d. The pH was 7.6 and the temperature was 25°C. The biological oxygen demand over 5 d (BOD_5) was 16 mg/L (Final effluent monitoring results from Weyerhaeuser Canada, Ltd.).

The presence of bacteria in the 1:1 floc was unquestionable. The SEM revealed numerous colonies in all samples taken at various time intervals (see Figures 9, 10 and 11). It was hoped to see an increase in EPS over the 52 h, however this was not evident. In all samples the bacteria did appear to be coated with a slime-like layer, determined by a deviation from their usual smooth shape. Any increase in the amount of EPS was undetectable from the SEM photographs alone.

Figure 9: SEM Photograph of Filtered Sample at 4.5 h

Figure 10: SEM Photograph of Filtered Sample at 28 h

Figure 11: SEM Photograph of Filtered Sample at 52 h

The majority of the bacteria appear to be cocci in shape, although a few spirilla were present. The size ranged from approximately 0.5 μm to 1 μm. Long fibers were also present.

The samples of the PME alone and RW also revealed the presence of bacteria (see Figures 12 and 13). Although SEM is not a quantitative technique, it was evident that much higher numbers of bacteria were present in the PME sample than the RW sample. The PME sample had large numbers of cocci present, usually < 1 μm in diameter. Some spiral-shaped bacteria were present as well as some long fibers.

Much time and effort was required to locate bacterial clusters from the upstream RW sample. The majority of the bacteria were cocci and slightly smaller than the bacteria in the PME sample. The river water bacteria appear more coated than the PME bacteria, indicating that EPS production could be a survival mechanism in an oligotrophic environment (i.e. the river). In the RW photograph, it is difficult to distinguish between any bacteria that might be present and the plain filter, shown in Figure 14.

Figure 12: SEM Photograph of PME Sample

Figure 13: SEM Photograph of RW Sample

Figure 14: SEM Photograph of Filter

The remaining two samples were taken at the Weldwood site in August 1998. Again, characteristics of the RW were not measured but data is available for the PME. On the day of sampling, the TSS was 20 mg/L, and discharged at a rate of 2708 kg/d. The pH was 8.0 and the temperature was 35°C (Final effluent monitoring results from Weldwood of Canada Ltd.). These are fairly normal typical values for the month of August.

The samples taken upstream of the pulp mill discharge at the Weldwood site were remarkably similar to the RW sample taken from the Wapiti River. The difference in sampling procedures was that the upstream sample was taken in the field, in a sterile container, while the RW sample was shipped to the laboratory in a non-sterile bucket. The bacteria were present in clumps; another possible survival mechanism; and they appear coated again.

A common structure was observed in many of the photographs: a centre sphere with star-like appendages. This can be seen in the middle of Figure 9 and the top right corner of Figure 12. It has also been enlarged in Figure 15. In both cases, it appears the appendages are directed toward bacteria. The centre sphere may actually be a bacterium,

however it is difficult to tell. It is possible that the appendages are of bacterial origin, contributing to flocculation.

Figure 15: Star-like structure present in SEM photographs

Dimensions of photograph are 5 x 7 μm.

The samples taken at 100 m and ~2 km downstream were quite similar, and not too different from the upstream sample. Since the approximate river velocity at the time of sampling was approximately 1.5 m/s (measured in field), a distance of 2 km corresponds to a flow time of about 22 min. Since biological flocculation is a delicate process that occurs after about 1 to 2 d (Muschenheim et al., 1989), the similarity should not be a surprise. Also, the Athabasca River is a large, fast-flowing river, an environment that may not be conducive for bioflocculation.

Figure 16: SEM Photograph of Upstream Bacteria

Figure 17: SEM Photograph of Bacteria ~2 km downstream

4.2 Heterotrophic Cultures

Much growth appeared on the heterotrophic plates after 48 h. Since filtration and incubation did not occur within 24 hours of initially taking the PME and RW samples from their source, quantitative results are not accurate. Filtration and incubation did occur within 96 h, so it was assumed that the types of bacteria would not change dramatically.

In general, the RW plates had a bacterial community that was less abundant and had less variety than either the PME or 1:1 mixture plates. Each river had a characteristic fauna. On R2A medium, the Athabasca River (at Weldwood) had a group of mostly red colonies that were culturable (Figure 18), while the Wapiti River (at Weyerhaeuser) had few red and mostly white or clear colonies (Figure 19). On SPAME, only the Wapiti RW was plated and a visible different fauna was present (Figure 20). No red colonies were visible, only white, opaque ones. RW was not plated onto the PME50 plates.

Figure 18: Athabasca RW plated on R2A, isolated from the liquid phase

Figure 19: Wapiti RW plated on R2A, isolated from the liquid phase

Figure 20: Wapiti RW plated on SPAME, isolated from the liquid phase

The PME samples contained a much wider variety of bacteria. The Weldwood effluent, plated on R2A produced a fauna of mostly white bacteria, with a few coloured ones in the less diluted sample (Figure 21). More coloured colonies were present in the floc samples than the liquid samples. The Weyerhaeuser plates were quite similar, but generally seemed to have a wider variety of bacteria. In Figure 21, it appears as if there are square colonies present. These are actually a result of the membrane filter that is divided into small squares.

Figure 21: Weldwood Effluent plated on R2A, isolated from the floc material

The major difference with the SPAME plates was that the bacteria took a longer time to grow. After 48 hours, which was ample time for growth on R2A plates, virtually no colonies were present on the SPAME plates. After 3 days, a wide variety of colonies began to appear, but the fauna differed substantially from the R2A plates (see Figure 22). An abundance of orange colonies were present that were not present on the R2A plates.

Figure 22: Weyerhaeuser Effluent plated on SPAME, isolated from the floc material

Slower growing still, were the colonies on the PME50 plates, which produced a much different fauna (see Figure 23). After 5 d of growth, the bacterial colonies were tiny and appeared white (but this may be because they were so small, or a result of the medium). A reasonable explanation for the slow growth on these plates is that the nutrients are not ready to be consumed, as they are in the richer, commercial media. The bacteria must break down the molecules into smaller, usable compounds, before any growth occurs. Also, there may be fewer nutrients overall. There were no major differences between the liquid and floc plates.

Figure 23: Weyerhaeuser Effluent plated on PME50 (5d growth), isolated from the floc material

The plates from the 1:1 mixture of PME and RW were more similar to the PME plates than the RW plates (see Figures 24 and 25). The Weldwood sample had mostly small, white colonies, while the Weyerhaeuser sample had a mixture of white and red colonies. This is interesting since it was the RW at Weldwood that had the abundance of red colonies. A large variety of bacteria was present from the flocculated material, from both Weldwood and Weyerhaeuser effluents.

Again, the colonies on the SPAME plates were slower growing. After 5 d, many large, orange and yellow colonies were present that were not present on the R2A plates. These colonies resembled those on the Weyerhaeuser effluent SPAME plates (see Figure 22). The growth on the plates from the 1:1 mixture of PME and RW resembled those from the effluent on the PME50 plates, with numerous tiny white colonies present after 5 d of growth.

Figure 24: Weldwood 1:1 mixture plated on R2A, isolated from the liquid phase

Figure 25: Weyerhaeuser 1:1 mixture plated on R2A, isolated from the floc material

4.3 Bacterial Isolation and Identification[1]

Thirty-six colonies were isolated from the heterotrophic plates (22 on R2A and 18 on SPAME) and re-plated onto the same solid medium. Isolation of bacteria from the PME50 plates was attempted but they died after the second transfer, thus none of the isolates were from the PME50 plates. The isolates and their respective sources are tabulated in Table 3. The "liquid" represents that they were isolated from the liquid portion and "floc", from the floc portion. All isolates were from the Weyerhaeuser effluent or river water, unless specified by WW, signifying they were isolated from the

Table 3: Isolates, their Sources and a Description

Isolate	Source	Description (on corresponding medium)
A	WW 1:1 liquid	white, round, slow-growing, ~2 mm diameter after 3 d growth
B	WW 1:1 floc	bright, yellow colonies, very slow-growing, well-defined, ~1 mm diameter after 2 d growth
C	WW 1:1 liquid	white, round, slow-growing, ~2 mm diameter after 3 d growth
D	WW 1:1 liquid	white, round, slow-growing, ~2 mm diameter after 3 d growth
E	1:1 liquid	white
F	WW PME liquid	white
G	WW PME liquid	pale yellow
H	PME liquid	bright yellow
I	WW RW	pink colonies, ~5 mm diameter after 2 d growth, flat colonies
HA	1:1 liquid	bright yellow
HB	1:1 liquid	yellow, ~2 mm diameter after 2 d growth
HC	1:1 liquid	off-white, ~3 mm diameter after 1 d growth, variable size
HD	1:1 liquid	off-white-clear, ~1 cm after 2 d growth, flat but raised in middle,
HE	1:1 liquid	creamy white, various sizes, <1 mm-2 mm diameter after 2 d growth
HF	1:1 liquid	white, tiny colonies, <1 mm diameter after 2 d growth
HG	1:1 liquid	white, ~2 mm after 2 d growth

[1] The references in this section are from Bergey's Manual of Determinative Bacteriology, 9th edition (Holt et al., 1994), unless otherwise noted.

Table 3 (cont.): Isolates, their Sources and a Physical Description

Isolate	Source	Description (on corresponding media)
HH	RW	white, ~ 1mm diameter after 1 d growth
HI	PME liquid	tiny, yellow colonies, ~1 mm diameter after 2 d growth, slow-growing
HJ	1:1 floc	white/clear colonies, ~1 diameter after 2 d growth, slow-growing
HK	1:1 floc	tiny, white-clear colonies, <1 mm diameter after 2 d growth
HL	1:1 floc	clear colonies, ~1 mm diameter after 3 d growth, irregular edges, flat
HM	1:1 floc	white-clear colonies
SA	RW	pale yellow colonies, ~2 mm diameter after 2 d growth
SB	RW	yellow, 2-3 mm diameter 2 d growth, round with peak in middle
SC	RW	white-clear, oval-shaped, ~3 x ~2 mm after 1 d growth, lots of mucous
SD	RW	yellow, tiny, <1 mm diameter after 1 d growth, irregular size, shape
SE	1:1 liquid	yellowish colonies, ~1 mm diameter after 1 d growth, peak in middle
SF	1:1 floc	yellow-beige, peaked in middle, ~5 mm diameter after 1 d growth, lot of mucous
SG	1:1 floc	yellowish, ~1 mm diameter after 1 d growth, not perfectly round
SH	1:1 floc	creamy-yellow, v. spread out, jagged edges, ~ 5 mm after 1 d growth, lots of mucous
SI	1:1 liquid	pale yellow, 1-2 mm diameter after 3 d growth, smooth edges, peaked in middle
SJ	1:1 liquid	yellow-orange, very slow-growing, ~1 diameter after 3 d growth, convex
SK	1:1 liquid	white
SL	1:1 liquid	orange
SM	1:1 liquid	off-white
SN	1:1 liquid	yellow-orange, very slow-growing, ~1 diameter after 3 d growth, convex

Weldwood combination. Those isolates starting with an "S" were isolated on SPAME. The cultures had to be frozen at this point, in a glycerol solution, due to time constraints. Not all species survived the freezing; there were 27 that survived. Gram staining, the oxidase test, the catalase test, a TSI test and growth on MAC plates were used to

characterize these cultures. The results are summarized in Table 4. Observation under a light microscope following the Gram stain indicated that most of the bacteria were bacilli in shape, a few being C- or L- shaped. All were Gram negative, which is characteristic of bacteria in PME (Fulthorpe et al., 1993). The Gram stain was not performed on bacteria that grew well on MAC plates, a medium that is selective for Gram negative, lactose-fermenting bacteria, since it could be assumed that they were Gram negative.

Table 4: Summary of Characterization Studies

ID	BAP	MAC	Gram stain and Appearance	oxidase	catalase	TSI	Comments	Growth ability
A	g (48), tiny, grey	ng (72)	g-b, curly	+	+	NC/NC		poor
B	ng (96)	ng	g-b, curly	-	-	ng (48)	grows well on R2A	poor ⊕
C	grey, opaque (fi, 48)	ng	g-b, curved, pleo, var. thickness	+	+	NC/NC		poor
D	grey, opaque (fi, 48)	ng	same as above	+	+	NC/NC (72)		poor
E	ng	ng						
F	ng	ng						
G	ng	ng						
I	small, opaque	ng (72)	g-b, in chains, mostly 2's	-	+	ng (72)	grows well on R2A	poor ⊕
HB	g (48), tiny	ng (72)	g-b, curly, chains	+	-	ng (48)	grows well on R2A	poor ⊕
HC	colif, i	pk (48)		-	+	A/A	late lactose fermenter	good
HD	small, grey	ng (72)	g-b, mostly single	+	+	Alk/NC		poor ⊕
HE	ng (72)	ng (72)	g-b, tiny, single rods	+	+	ng (48)	grows on R2A	good
HF	tiny, grey	wt (48)	g-b, single, some chains	+	+	NC/NC		poor ⊕
HG	tiny (48)	nh (48)	g-b, mostly single	+	+	Alk/NC (48)		poor
HH	tiny, grey	ng (24)	g-b, thin rods	+	+	Alk/A		good
HI	ng (48)	ng (48)	g-b, curly	+	-	ng (48)	grows on R2A	poor ⊕
HJ	small grey (48)	ng (48)	g-b, tiny, single rods	+	-	Alk/alk		poor ⊕
HK	tiny (24)	ng (24)	g-b, thin rods	-	+	Alk/NC (48)		poor
HL	tiny (24)	ng (24)	g-b, single rods	+	-	Alk/NC (48)		poor
SA	tiny, opaque (48)	wt (48)	g-b, single	+	+	Alk/NC		poor ⊕

Table 4 (cont.): Summary of Characterization Studies

ID	BAP	MAC	Gram stain and Appearance	oxidase	catalase	TSI	Comments	Growth ability
SB	tiny, opaque (24)	ng (24)	g-b, single, short chains	-	+	NC/NC (48)		poor ⊕
SC	colif, I	wt		+	+	Alk/NC		good
SD	ng (72)	ng (72)	g-c	-	+	ng (48)	grows on R2A	good
SE	colif, h	wt		+	+	Alk/NC		good
SF	colif, h	wt		+	+	A/A	late lactose fermenter	good
SG	colif, i	wt		+	+	Alk/NC		good
SH	colif, h (48)	wt	g-b, single rods	+	+	Alk/NC		poor ⊕
SI	tiny (24)	ng (24)	g-b, tiny rods	+	+	NC/NC (48)		poor
SJ	ng (48)	ng (48)	g-b, in clumps	-	+	ng (96)	grows on R2A	poor ⊕
SK	ng	ng						
SL	ng	ng						
SN	ng (48)	ng (48)	g-c, single	-	+	ng (72)	grows on R2A	poor ⊕

Abbreviations: g, growth (number of hours given in brackets); ng, no growth; g-b, Gram negative bacilli; g-c, Gram negative cocci; pleo, pleomorphic (variable length); h, hemolytic; i, indifferent; fi, fine indifferent; colif, coliform; wt, white; pk, pink, A, acid; Alk, Alkaline; NC, No change.

From the data in Table 4, all bacteria isolates were classified as GN-NENT, except for two: SF and HC, due to their acid/acid reactions with the TSI slant. These were classified as GN-ENT and were treated separately.

The Biolog GN2-Microplates™ performed fairly well at giving a positive ID, with affirmative identification for 13 of the 27 isolates. The results are shown in Table 5. Nine species gave false positive results (marked with a * in Table 5), which resulted in all wells being purple. This was not surprising because false-positive results are commonly observed with *Klebsiella*, *Enterobacter* and *Serratia* strains (Biolog Manual, 1999) and *Klebsiella* spp. have been found in PME (Fulthorpe et al., 1996). The GN2-MircoPlates were re-inoculated, with the addition of sodium thioglycolate, an anti-capsule agent. The majority of these produced readable fingerprints, however one, SD, still gave a false positive result. This may be because SD is actually Gram positive, or oligotrophic Gram negative, neither of which are suitable for the GN2-MicroPlates. Because no fingerprint was obtained, no estimation of the species can be made.

Table 5: Summary of Biolog Identification

ID	Biolog ID	ID	Biolog ID
A	no ID, ressembles D in appearance and biochemical tests	HK	*Aquaspirillum metamorphum*
B	*Roseomonas* genomospecies 6	HL*	no ID, *possibly Lampropedia hylina*
C	no ID, ressembles D in appearance and biochemical tests	SA	*Brevundimonas vesicularis*
D	*Agrobacterium* like-cystic fibrosis	SB	*Flavobacterium marinotypicum*
I*	*Pasteurella pneumotropica*	SC	*Comamonas testosteroni*
HB*	*Achromobacter cholinophagum*	SD**	
HC	no ID, likely *Enterobacter* spp.	SE	no ID, likely *Pseudomonas* spp.
HD	*Acidovorax delafieldii*	SF	no ID, possibly *Aeromonas* spp.
HE	*Acidovorax delafieldii*	SG	*Bordetella bronchiseptica*
HF	*Aquaspirillum metamorphum*	SH	no ID, likely *Pseudomonas* spp.
HG*	no ID	SI*	no ID, possibly *Pasteurella trehalosi*
HH	no ID, but likely belonging to *Pseudomonas* spp.	SJ	no ID, likely *Pseudomonas aurantiaca*, resembles SN
HI*	no ID, possibly *Aquaspirillum putridiconchylium*	SN	no ID, likely *Pseudomonas aurantiaca*, resembles SJ
HJ*	*Pseudomonas echinoides*		

The Biolog software generates a list of the top ten possible species for each fingerprint. For each, the probability, SIM and the type are given. This tabulated information can be found in Appendix D and a discussion follows.

Both HF and HK were identified as *Aquaspirillum metamorphum*. *A. metamorphum* has been characterized as slow-growing, oxidase positive and it will grow on TSI but not on MAC plates. These characteristics do not seem to fit with those reported for HF and HK. HF was slow-growing, but growth did appear on the MAC plate after 48 h. HK did not grow on the MAC plates, but was oxidase negative (Table 4). However, upon re-testing, this reaction was positive but very slow. In Bergey's Manual (Holt et al., 1994), where the above characteristics were taken from, a reaction is considered positive if 90% or more of the strains react positively. Likewise, a negative reaction refers to 90% or more of the strains reacting negatively. Thus the identification cannot be rejected on this fact alone. *A. metamorphum* has been isolated from a large

variety of fresh water sources, especially stagnant areas with a high organic content (Holt, et al., 1994). So it would not be unreasonable to find *A. metamorphum* in pulp mill effluent, or river water. (Both were isolated from a 1:1 mixture, HF from the liquid portion and HK from the floc portion.)

Another *Aquaspirillum* species may have been present in the PME at Weyerhaeuser. HI had no ID, but *A. putridiconchylium* was at the top of the list with a SIM of 0.35. (A SIM of 0.5 is required for positive identification.) *A. putridiconchylium* is characterized as weakly catalase positive or negative, oxidase positive, no growth on MAC plates and growth on TSI slants. All is consistent with HI, except no growth was observed on the TSI slant (Table 4).

Two species HD and HE were identified as *Acidovorax delafieldii*. A previous study (Fulthorpe et al., 1993) had isolated *Acidovorax* spp. from PME. The *Acidovorax* genus was created in 1990, and described as straight or slightly curved rods, 0.2 to 0.7 µm by 1.0 to 5.0 µm, occurring singly or in short chains. Three species were mentioned in Bergey's Manual (Holt et al., 1994), including *A. delafieldii*. All species are oxidase positive, consistent with HD and HE. However HD and HE were not exactly identical. Both were oxidase positive, catalase positive and did not grow on MAC plates. But HE did not grow on the blood agar plates (BAP) or the TSI, and HD did. So it is unlikely that both isolates are *A. delafieldii*.

Numerous *Pseudomonas* spp. were identified in this study, as in the previous study by Fulthorpe et al. (1993). However, the exact species was rarely able to be determined. HH, SE, SH, SJ and SN likely belong to the genus *Pseudomonas* and HJ was identified as *P. echinoides*. *Pseudomonas* spp. prefer environments where the pH is close to neutral, there is much organic matter, a mesophilic temperature and lots of dissolved oxygen (Holt, et al., 1994), an environment similar to a treatment lagoon. However *P. echinoides* in particular, has been isolated as a laboratory contaminant, so it is impossible to say whether it was actually present in the PME, or is the result of contamination of the sample. SJ and SN could not be identified by the Biolog method, but *P. aurantiaca* was top of the list, with SIMs of 0.33 and 0.41 respectively. However *P. aurantiaca* is oxidase positive and both SN and SJ were oxidase negative (Table 4).

All *Pseudomonas* spp. are catalase positive and all of the isolates identified as *Pseudomonas* spp. were, except for HJ. Upon re-testing, HJ was found to be very weakly positive. The oxidase reactions are variable. HH had a positive ID, as *Pseudomonas* Group 2 (*Burkholderia*-like). According to Ochi (1995), the *Burkholderia* genus is very similar to the *Pseudomonas* genus, differing in their ribosomal proteins. SE had no ID, but listed only *Pseudomonas* spp. as possible IDs, and SH listed the top three possibilities as *Pseudomonas* spp.

Isolate SG was identified as *Bordetella brochiseptica*, a minute coccobacillus usually found singly or in pairs. It is characterized as oxidase positive and is red on MAC plates, indicating its ability to ferment lactose. SG is oxidase positive, but produced white colonies on the MAC plate, representative of non-lactose fermenting organisms. *B. brochiseptica* is a highly pathogenic organism, infecting both humans and animals world-wide. Its preferred environment is the epithelial cells lining the upper respiratory tract of domestic and wild animals. While it may be possible that *B. brochiseptica* be found in a natural setting, it seems unlikely that it would flourish in an environment such as a treatment lagoon. The identification may be erroneous, or it could be the result of laboratory contamination.

Both SN and SJ had another *Bordetella* spp. listed as a second possibility. Their first possibility was belonging to the *Pseudomonas* genus. In the fingerprint, conversion of a negative well to a borderline well, resulted in *Bordetella hinzii* appearing at the top of the list for SJ, with a SIM of 0.43 (compared to a SIM of 0.33 for the *Pseudomonas* spp.). Since the two fingerprints apparently resemble each other greatly, it would not be illogical to suggest SN and SJ as being a *Bordetella* spp., or perhaps SG as being a *Pseudomonas* spp. The second possibility for SG is *Pseudomonas alcaligenes*, with a SIM of 0.01. However with some conversion of positive and negative wells to borderline cases, a higher SIM could be obtained.

Isolate I was identified as *Pasteurella pneumotropica*, a rod-shaped bacterium, producing colonies 1.6 to 2.0 mm in diameter (48 h, 37°C) on BAP. This corresponds well to the characteristics observed for I. However, *P. pneumotropica* is parasitic in vertebrates, preferring the upper respiratory and digestive tract. Isolate I was common and abundant in the Weldwood river water samples, an unlikely characteristic of a

pathogen. Therefore, it may be misidentified, or be the result of laboratory contamination. *P. pneumotropica* is reported to be very similar to *Actinobacillus* spp., also present in the *Pasteurellaceae* family. However, from the list of differentiating characteristics in Bergey's Manual, it points to I being *P. pneumotropica* in all tested cases.

There may have been another *Pasteurella* species present as well, in the 1:1 mixture of PME and RW. SI had no ID but at the top of the list with a SIM of 0.22, was *Pasteurella trehalosi*. SI somewhat fits the description of the facultative anaerobic *Pasteurella* genus as well: rod-shaped and oxidase positive. Most *Pasteurella* species are catalase positive, but *P. trehalosi* is characterized as catalase negative. SI was catalase positive. *Pasteurella* spp should produce an acid reaction with the TSI slant. For *P. trehalosi*, this reaction is delayed, which may be why it was not noted for SI. I did not grow on the sugars at all. Looking at these biochemical characteristics it seems possible that a *Pasteurella* species was present in the river water and/or effluent. However, the fact that *Pasteurella* species prefer the mucous membranes of the upper respiratory tract of mammals leads one to believe that I and SI may have been misidentified.

Of all the isolates, only two were classified as enteric: HC and SF, due to their acid/acid reaction with the TSI slant. These were treated under different conditions (35°C, 4 to 6 h incubation) as specified by Biolog. *Escherichia coli* was used as a control since it was available and would give an indication of the success of the system. All three species resulted in an uncertain identification. A SIM of 0.75 is required for enterics, thus the fingerprint must be more precise. For the control, *E. coli*, the correct ID was on top of the list with a SIM of 0.67. The second species on the list was *E. coli* as well, but of a different strain. Therefore this shows that it is not unreasonable to suggest that the species on top of the list may be the correct ID.

The top four species on the list for HC were of the *Enterobacter* genus; the first with a SIM of 0.69. Thus it can be assumed that HC belongs to the *Enterobacter* genus, possibly being *E. asburiae*. *Enterobacter* spp. are straight rods that ferment glucose with the production of acid and a gas. The TSI reaction of HC indicated production of acid (by the yellow colour) but the gas was uncertain. Production of a gas is usually indicated by a gas bubble forming at the bottom of the test tube that pushes the agar slant upwards. However, if there is a crack in the agar, the gas can escape. This was the case for HC,

explaining the "questionable gas production" (?gas) noted in Table 4. *Enterobacter* spp. are widely distributed in nature, so after analysis it seems logical that HC is an *Enterobacter* spp. with no evidence indicating otherwise. They are also found in freshwater, sewage, soil, plants and feces.

SF also had no positive ID. However all of the top ten species listed were non-enteric. There were not enough additional GN2-MicroPlates to re-test SF under non-enteric conditions, so the ID remains inconclusive. At the top of the list was an *Aeromonas* spp. with a SIM of 0.39. If SF is actually a non-enteric bacteria, some of the reactions may have an initial lag phase and require the full 16 to 24 hours of incubation. SF fits the description of the *Aeromonas* spp. (oxidase and catalase positive) and *Aeromonas* spp. are found on fresh water, sewage and sludge. Also of interest is that SF was recorded as a late lactose fermentor, indicated by the delayed acid/acid reaction of the TSI slant. *Aeromonas* spp. are usually lactose negative, but a few strains may develop lactose fermenting abilities.

Isolate D gave a positive ID for *Agrobacterium* like-cystic fibrosis. *Agrobacterium* are rods, occurring singly or in pairs, present all over the world in soils, especially the rhizosphere of plants. Growth on carbohydrate-containing media is usually accompanied by copious amounts of extracellular polymeric slime. Bacteria from D were curved, pleomorphic rods of variable thickness. Colonies grew very slowly on BAP but well on R2A. *Agrobacterium* could be present in the PME or RW (D was isolated from the 1:1 mixture) but the physiological description does not seem to match.

During the isolation and characterization studies, it was noted that all of A, C and D behaved similarly: their growth behaviour was identical, as was their appearance, shape and oxidase and catalase reactions. As well, their fingerprints were extremely similar, differing by the reaction in one or two wells. Neither A, nor C had a positive identification. Examining the top ten species for A and C, *Agrobacterium* like-cystic fibrosis is found at #3 and #5 respectively. Both A and C had a *Pseudomonas* species at the top of the list, and D had a *Pseudomonas* species listed at #3. A, C and D could be *Pseudomonas* species, since the *Pseudomonas* genus consists of slightly curved rods, that are oxidase and catalase positive. However this information is too vague to conclude for certain.

Under the assumption that A, C and D were all the same species, a combined fingerprint was produced (using the most common reaction in each well). When entered into the computer, no ID was found, but *Agrobacterium* like-cystic fibrosis was at the top of the list and a *Pseudomonas* species was second.

Fulthorpe et al. (1993) mentioned a group of unidentified Gram negative methylotrophs (organisms capable of growth on C_1 compounds), common among the culturable isolates from the treatment system. These were characterized as oxidase and catalase positive, pleomorphic and irregularly thickened rods or L- or C- shaped cells. Some of these had deep yellow pigment and some had a pale orange pigment. A, C and D were white, but the rest of the description seems to match perfectly. The pigment may be a result of the media used. Thus is appears likely that A, C and D belong to this unidentified group.

Isolate SC was identified as *Comamonas testosteroni*, with a SIM of 0.71. Second on the list was another *Comamonas* spp., *C. acidovorans*. The *Comamonas* genus was created in 1987 and included the re-classification of *Pseudomonas testosteroni* as *C. testosteroni* (Tamaoka et al., 1987). The *Comamonas* genus consists of straight or slightly curved rods, occurring singly or in pairs. It is oxidase and catalase positive, agreeing with observations of SC. No environment was given in Bergey's Manual (Holt et al., 1994) but since it is similar to *Pseudomonas* spp., it may have a similar environment. Since no observations seem to contradict the conclusion, it is likely that SC is *C. testosteroni*.

SA was identified as *Brevundimonas vesicularis*. This genus was created in 1994 (Segers et al., 1994), from a *Pseudomonas* spp: *P. vesicularis*. The reclassification was based on DNA-rRNA hybridization studies. Since these species were thought to be in the *Pseudomonas* genus for so long, basic characteristics are likely similar: straight or slightly curved rods, catalase positive and prefer neutral environments with a high organic content. SA is catalase positive, with a bacilli shape, so the observations do not contradict with the description. Therefore, SA may be *B. vesicularis*.

HB was positively identified as *Achromobacter cholinophagum*. The 1994 edition of Bergey's Manual has no mention of the genus, however it was been mentioned in the literature by Poinar (1967), who investigated use of an *Achromobacter* species for insect control and by Bieszkiewicz et al. (1998), who attempted to use an *Achromobacter* strain

to treat petroleum products. In Bacterial Systematics (Logan, 1994), *Achromobacter* is mentioned as one of seven possible names for the *Acinetobacter* genus. According to this source, many independent isolations of the genus have led to the wide variety of names. *Acinetobacter* refers to the oxidase negative strains and *Achromobacter* refers to the oxidase positive strains (Logan, 1994). HB is oxidase positive, so at least this is consistent with the literature. *Acinetobacter* spp. have already been isolated from pulp mill effluent (Fulthorpe et al., 1993) so it is not surprising at this result. What is curious is that there is no mention of the *Achromobacter* genus in Bergey's Manual (Holt et al., 1994). Members of the *Acinetobacter* genus are oxidase negative and catalase positive, and HB is oxidase positive and catalase negative. Both of *Acinetobacter* and *Achromobacter* spp. are glucose non-fermenting, gram negative bacteria.

The *Roseomonas* genus, of which B was identified, was isolated from blood in 1993 (Rihs et al., 1993) and did not fit into any other previously described species. Six species were isolated at this time, names assigned to the first three, and the others are known only as genomospecies 4, 5 and 6. All are pink-pigmented bacteria. Isolate B was yellow-pigmented, and produced tiny, well-defined colonies on R2A agar. It would not grow on the BAP or MAC. *Roseomonas* spp. are catalase positive and oxidase variable (similar to B), but they will grow on sheep's blood agar and MAC plates. The only isolate of *Roseomonas* genomospecies 6 was isolated from a breast incision (Rihs et al., 1993). From all this evidence, it appears unlikely that B is *Roseomonas* genomospecies 6. Second on the list is another *Roseomonas* spp. but third and fourth are *Pseudomonas* species. However the SIM this far down the list is 0.00, thus the identification remains uncertain.

SB was identified as *Flavobacterium marinotypicum*. *Flavobacterium* spp. are rods with parallel sides and rounded ends, oxidase positive, catalase positive and are usually orange-yellow pigmented. They are widely distributed in soil and water (Holt et al., 1994). SB was yellow in colour (on SPAME) but oxidase negative. The second possibility was listed as *Achromobacter cholinophagum*. The *Achromobacter* genus is considered to include the oxidase positive strains of the *Acinetobacter* genus. This is still not consistent with SB. The oxidase reaction was re-tested and may be considered weakly

positive, so this particular strain may be anomalous. If this is the case, SB could be *F. marinotypicum*.

Recently six new species were proposed in the genus *Microbacterium* (Takeuchi and Hatano, 1998). Based on DNA-DNA hybridization, *F. marinotypicum* was transferred to this genus as *M. maritypicum*. comb. nov. The Biolog Gram negative database was last revised in December 1998, so it may not contain this change. According to Takeuchi and Hatano (1998), *M. maritypicum* are yellow-pigmented, Gram positive rods. *Flavobacterium* are Gram negative, but Takeuchi and Hatano (1998) performed an analysis of the cell wall components and based on this, must have reclassified it as Gram positive. SB consists of yellow-pigmented rods, so this correlates well.

After addition of the thioglycolate, the fingerprint for HG was still not obvious. All wells were purple, with little differentiation. A fingerprint was recorded, however it was quite ambiguous. No ID was found, with the highest SIM of 0.27 belonging to *Sphingomonas macrogoltabidus*. Second on the list was an *Acinetobacter* spp., with a SIM of 0.13. Both of these are possible, but with the ambiguous fingerprint, it cannot be concluded for certain.

HL had no definite identity, but at the top of the list, with a SIM of 0.49, was *Lampropedia hyalina*. Upon verification in Bergey's Manual (Holt et al., 1994), it seems unlikely that this is the case. The most distinguishing feature of the *Lampropedia* genus (of which *L. hyalina* is the only species) is that it comes in sheets of rounded, almost cubical cells, arranged in squares of 16 to 64 cells. This well-defined structure cannot be mistaken for the single rod shape of HL. Isolation of *L. hyalina* has shown that it does prefer an environment rich in organic matter. Second on the list, with a SIM of 0.39 was *Pseudomonas echinoides*, a species which has been isolated as a laboratory contaminant. It seems much more likely that HL is this *Pseudomonas* species, than the highly structured *L. hyalina*.

From this analysis, it can be concluded that some species were likely present in the effluent and/or river water. They were *Aquaspirillum* spp., *Acidovorax* sp., *Pseudomonas* spp., *Enterobacter* spp., *Comamonas* spp., *Brevundimonas* spp., *Flavobacterium* spp. and *Achromobacter* spp. Two other species could possibly have

been present as well: *Pasteruella* spp. and *Aeromonas* spp. The identification of the remaining isolates was likely erroneous because of the reasons for each case, discussed above.

4.4 Re-Introduction into Sterile Effluent

Two methods of sterilization were evaluated: successive autoclaving and the addition of chloroform. The addition of chloroform did not sterilize the effluent, perhaps due to the large number of bacteria. Successive autoclaving was successful, provided there was no contamination. Thus successive autoclaving was chosen as the method of sterilization.

The particle size distributions revealed that autoclaving the effluent greatly increases the number of tiny particles (see Figure 26). Since this seemed to be the only efficient method of obtaining sterility, the experiment had to be continued despite this difference. The sterile control would be valuable in determining if flocculation did occur, since flocculation should decrease this number of tiny particles, despite the increase in individual bacteria and possibly increase the number of larger particles. The control inoculated with a sample of pulp mill effluent should give a realistic idea of the total extent of flocculation that is achievable under these conditions. The purpose of the untreated control was to indicate how the test conditions differed from the actual conditions. The untreated control does not realistically simulate the river environment, however its physical and chemical composition would be more representative of the actual solutions. All runs of the controls are tabulated in Appendix G.

The sterile control exhibited very little flocculation. There was a slight decrease in the particles sized 2 to 4 μm (indicated by $C/C_0 < 1$ in Figure 27). This can be attributed to any physical/chemical flocculation that might have occurred. However, it must be remembered that this is not indicative of the actual extent of physical/chemical flocculation since autoclaving the samples likely altered the physical/chemical properties of the effluent and river water. There was also a slight decrease in turbidity (see Table 6), likely due to physical/chemical flocculation as well. In addition, there was no increase in the larger sized particles for the sterile control. However, these particles may have grown

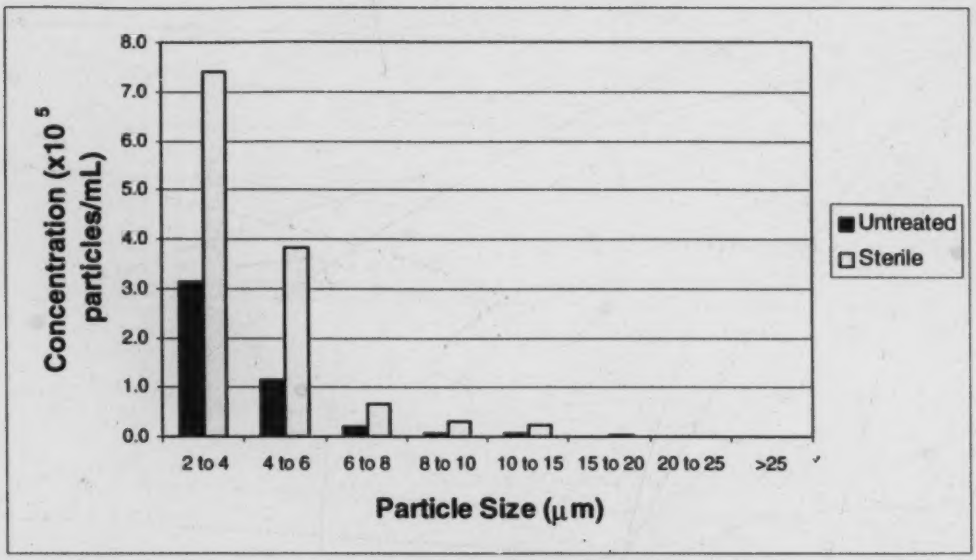

Figure 26: Effect of Autoclaving Pulp Mill Effluent on its Particle Size Distribution

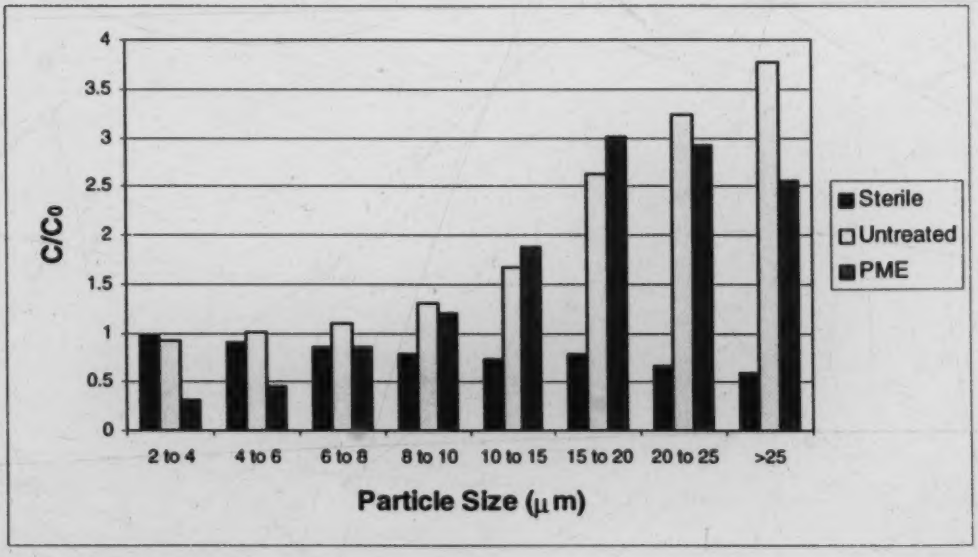

Figure 27: Comparison of the Three Controls

C_0 is the initial concentration of particles size 2 to 4 μm and C is the concentration after 4 d

large enough to settle out and accumulated on the bottom, as is representative of type 2 settling. At the end of 4 d, most flasks had sediment on the bottom.

The accumulation of sediment on the bottom of the flask was chosen to be ignored. It should not change the concentration of particles sized 2 to 4 μm in solution, which were used as a measure of flocculation in this experiment. The accumulation of sediment, by itself, cannot be a measure of flocculation, as some of it may have been a result of type 1 settling. A more thorough approach would be to look at a mass balance equation of the suspended and settled solids, to determine their fate.

Six PME controls were tested, three with May effluent and three with July effluent. As a first remark, after 4 d, the PME control did not produce a dramatic difference in either the turbidity or particle size distribution. After 6 d, the change was visible. A possible explanation for the delay is that the inoculated bacteria in the effluent may not have been as healthy or as abundant as those introduced by test tubes, resulting in a longer lag phase. In addition, there would have been non-flocculating bacteria present, that could have clouded the solution by increasing the number of smaller particles. Despite this observation, the 4-d analysis results will be used in statistical analysis for comparison purposes and are shown in Figure 27.

The untreated control, a 1:1 mixture of non-sterile PME and RW, was considerably different. The initial turbidity reading and particle size distribution were much lower than either of the other two controls; obviously an effect from not being autoclaved. The overall change is not as dramatic as one might expect; a difference in turbidity is barely detectable. This may be because the effluent remained in the cold room for some time before testing and some flocculation might have already occurred.

To determine if there was a significant difference between the effluent and RW combinations on the different collections months, a two-mean t-test was used on the six runs of the PME control. Three runs were completed with the May sample, from the spring, where the sediment load in the river was quite high (although it was not measured). Another three runs were completed with July samples, where the river water was much clearer and the effluent was visibly darker. (Again, no standard measurements

were made.) A comparison was made for each particle size range in the particle size distribution. At the lower size ranges, the difference between the May and July samples was not significant. However, for the >25 µm and 20 to 25 µm size ranges, the difference was significant (at the 95% confidence level). The July sample produced significantly higher C/C_0 values than the May sample. The difference in turbidity was found to be insignificant as well ($t_{calculated} = 2.42$ and $t_{criticial} = 2.776$).

It was noted (although not measured) that at the end of 4 d, the flasks with May sample had much more sediment on the bottom than the July flasks. With the increased sediment load in the May sample, there may have been more initial sediment, that was of larger size and could settle out, without any flocculation (i.e. type 1 settling). The increased amount of sediment could also provide more substratum onto which bacteria could attach, grow and divide, and induce flocculation (i.e. type 2 settling). It is likely that a combination of both type 1 and type 2 existed. Consequently, during the settling process, the larger particles could interact with smaller ones and bring them down as well. This is a possible explanation as to why there were fewer large particles in the May sample than the July sample (i.e. C/C_0 was significantly higher for the July sample than the May sample, for the >25 µm and 20 to 25 µm size ranges).

For the individual isolate runs, a combination of samples was used, from both May and July. The results were all averaged together but the higher size range data are not used for comparison. A preliminary run for each isolate was completed with the May samples. The results were compared to the sterile control. Based on the turbidity readings, only three isolates produced a clear effluent: HC, SC and SF. The change in the turbidity for all isolates is shown in Table 6, with the above-mentioned isolates in bold. Since the initial turbidity varied slightly, a normalized value, T/T_0, is given for comparison purposes. Using a decrease in the number of smallest measurable particles present (2 to 4 µm in diameter) as another indicator of flocculation, four more species demonstrated flocculation abilities as well: HH, HJ, SG and HL. Since the initial number of particles varied with each run, these were normalized as well (C/C_0, where C is the number of particles size 2 to 4 µm in 1 mL). Figure 28 compares all isolates and controls based on their C/C_0 value for particles 2 to 4 µm in diameter. From this comparison, the

flocculating species are easily determined. (A comparison of the entire particle size distribution to the sterile standard, for all isolates can be found in Appendix E.)

Table 6: Change in Turbidities for all Species and Controls

Species	Initial Turbidity (NTU)	Final Turbidity (NTU)	T/T_0	Species	Initial Turbidity (NTU)	Final Turbidity (NTU)	T/T_0
A	160	237	1.48	HK	160	180	1.13
B	196	173	0.88	HL	222	270	1.22
C	153	170	1.11	SA	196	236	1.20
D	156	241	1.54	SB	217	89	0.41
I	281	168	0.60	**SC**	**130**	**-4**	**-0.03**
HB	282	59	0.21	SD	219	192	0.88
HC	**200**	**10**	**0.05**	SE	222	144	0.65
HD	293	230	0.78	**SF**	**165**	**-4**	**-0.02**
HE	223	175	0.78	SG	169	130	0.77
HF	200	178	0.89	SH	203	165	0.81
HG	223	179	0.80	SI	223	192	0.86
HH	190	40	0.21	SJ	204	233	1.14
HI	225	163	0.72	SN	218	215	0.99
HJ	223	57	0.26	sterile (ave)	-	-	0.93
PME (ave)	-	-	0.69	untreated (ave)	-	-	0.99

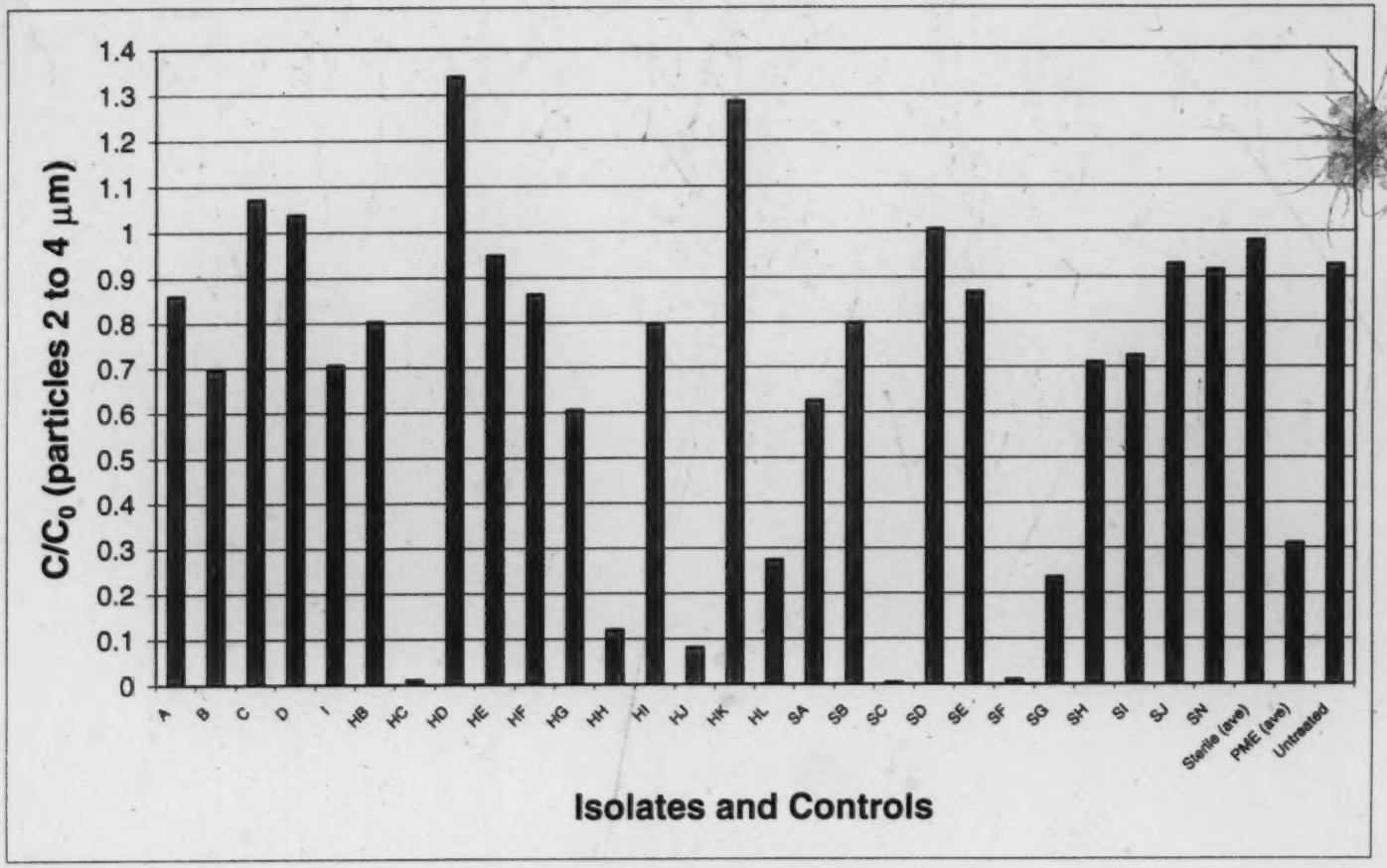

Figure 28: Comparison of all Isolates and Controls based on C/C_0 (2 to 4 µm)

C_0 is the initial concentration of particles sized 2 to 4 µm in diameter and C is the concentration after 4 d.

From this analysis, species were grouped into one of three categories: 1) enhanced flocculation abilities noted, 2) mild flocculation abilities noted and 3) no flocculation abilities noted. The third category included all the species that actually increased the number of small particles. These were HD, C, D, HK and SD. Seven species were classified as capable of enhanced flocculation: HH, HC, SF, SC, HJ, SG and HL. The remaining species showed a slight decrease in the concentration of small particles, but it was not vastly different from the sterile control.

It is interesting to note that there does not seem to be any common characteristic amongst the isolates capable of enhanced flocculation. All of HC, SF, SC and SG were considered good growers. HJ and HL were classified as poor growers and HH was classified as poor ⊕. SC and SF produced an abundance of mucous, but none of the other isolates did (on the initial solid medium used during the isolation experiments). Other isolates that were classified as good-growers or mucous-producers did not induce flocculation. There were no trends in the oxidase, catalase and TSI reactions. Thus flocculation abilities appear to be an independent trait.

For statistical purposes, the experiment for the isolates demonstrating enhanced flocculation abilities were repeated three more times, with effluent and RW samples taken in different months, May and July. These results varied greatly. The initial runs had been performed with an effluent grab sample taken at the end of May 1999. This sample was all used, so a new sample had to be ordered. The new RW was much clearer and contained less sediment (a visual observation only, as no standard tests were performed for comparison). The initial readings for both turbidity and particle size distributions were much lower. However, the results were normalized for comparison purposes, and all values were averaged together. A two-mean t-test was used to determine if the results differed significantly from the mean, summarized in Tables 7, 8 and 9.

Table 7: Average and Standard Deviation of C/C_0 and T/T_0 for the Repeated Runs of the Selected Isolates

Isolate	Number of runs	Mean (C/C_0 for 2 to 4 μm)	Std. deviation	Mean (T/T_0)	Std. deviation
HC	4	0.770	0.51	0.803	0.51
HH	2	0.453	0.47	0.465	0.36
HJ	4	0.871	0.55	0.784	0.40
HL	3	0.506	0.61	1.21	0.042
SC	4	0.931	0.69	0.668	0.48
SF	4	0.561	0.38	0.493	0.37
SG	4	0.849	0.52	0.919	0.12
Sterile	4	0.979	0.12	0.928	0.32

Note: C_0 is the initial concentration of particles sized 2 to 4 μm, C is the concentration of particles sized 2 to 4 μm after 4 d, T_0 is the initial turbidity reading and T is the turbidity reading after 4 d.

Table 8: Statistics Summary for Comparison to Sterile Standard, based on C/C_0 (2 to 4 μm), for the Data in Table 7

Isolate	Pooled variance estimate (s^2)	$t_{calculated}$	Degrees of freedom	$t_{critical}$ (95%)
HC	0.13725	0.80	6	2.447
HH	0.066025	2.37	4	2.776
HJ	0.15845	0.38	6	2.447
HL	0.15748	1.56	5	2.571
SC	0.24525	0.14	6	2.447
SF	0.0794	2.10	6	2.447
SG	0.1424	0.487	6	2.447

Table 9: Statistics Summary for Comparison to Sterile Standard, based on T/T_0, for the Data in Table 7

Isolate	Pooled variance estimate (s^2)	$t_{calculated}$	Degrees of freedom	$t_{critical}$ (95%)
HC	0.131	0.49	6	2.447
HH	0.0332	2.94	4	2.776
HJ	0.12998	0.56	6	2.447
HL	0.0621456	1.48	5	2.571
SC	0.116	1.08	6	2.447
SF	0.0690	2.34	6	2.447
SG	0.0584	0.053	6	2.447

First, looking at the C/C_0 parameter (2 to 4 μm), no isolates produced a significant response. This is because there was much variation among the runs and the standard deviation was very high. An example is SG, whose individual C/C_0 values were 0.236, 1.477, 0.685 and 0.999. This first value was obtained with May samples and the latter three with July samples. For all isolates classified as possessing enhanced flocculation abilities (with the exception of HL), the lowest C/C_0 value was obtained with the May effluent. The individual tests for these seven isolates are shown in Appendix F. Perhaps it was the high sediment load, present in the river water in May that aided in flocculation. As discussed earlier, the increased amount of sediment may have been larger in size and settled out independent of any reactions. Or the increased sediment could have provided more substratum available for bacteria attachment, promoting bacterial growth, development and potentially the excretion of polymers. It appears that the flocculation abilities of the bacteria are largely dependent on their environment. It has already been demonstrated that the production of EPS by the bacteria is dependent on their environment (Flemming, 1993; White, 1995) and EPS has been shown to aid in flocculation (Frieman and Dugan, 1968). Thus it seems logical that flocculation abilities depend on the environment of the bacteria. The nature of the pulp mill effluent will change with the type of wood being processed, the process being used and the treatment

of the effluent. The nature of the river water is more dependent on the seasons, with a high sediment load during the spring run-off and much lower during the rest of the year. Also, storage of the sample in the cold room will likely change physical/chemical properties, explaining the differences noted for one particular sample.

Looking at the T/T_0 values, only HH produced a significant response. However only two runs were used for this since the other two runs were impure. (They could not be repeated due to time constraints.) It is likely that if additional runs were performed, the results would vary, as they did for the other isolates, producing a higher standard deviation and render the HH results insignificant.

A similar explanation as that used for the C/C_0 values can be used to explain these. The flocculation abilities of the bacteria seem to be largely dependent on the nature of their environment, which includes the nature of the pulp mill effluent and the river water.

It would appear that there was no obvious correlation between the C/C_0 results and the T/T_0 results. To determine if this is true, a plot of C/C_0 vs. T/T_0 (Figure 29) was done, of only the first runs for all isolates. From this, there appears to be a general positive trend, with several outliers. A line of best fit has been added and some selected points labeled. It is interesting to note that there appears to be two distinct regions: those with $C/C_0 < 0.4$, including the selected isolates and the PME (ave) point, and those with $C/C_0 > 0.6$, including the remaining isolates and standards. There is no obvious T/T_0 division.

The line drawn on Figure 29 is only one possibility. It could be a straight line, originating at the origin, with a positive slope. It could also be a curved line, from the origin. The way it is drawn in Figure 29 indicates that these are two separate regions, possible identifying a difference between flocculating and non-flocculating species. Some of the points appear to be outliers that can be classified in two categories. An example of the first type is HL, with a very low C/C_0 value but a high T/T_0 value. This can be explained by realizing that the turbidity can be a result of the larger sized particles. HB is an example of the other type of outlier. It has a high C/C_0 value, but showed a high reduction in turbidity over the 4 d. In this case, the small particles (2 to 4 μm) are present in the final solution, but they are not contributing to the turbidity reading.

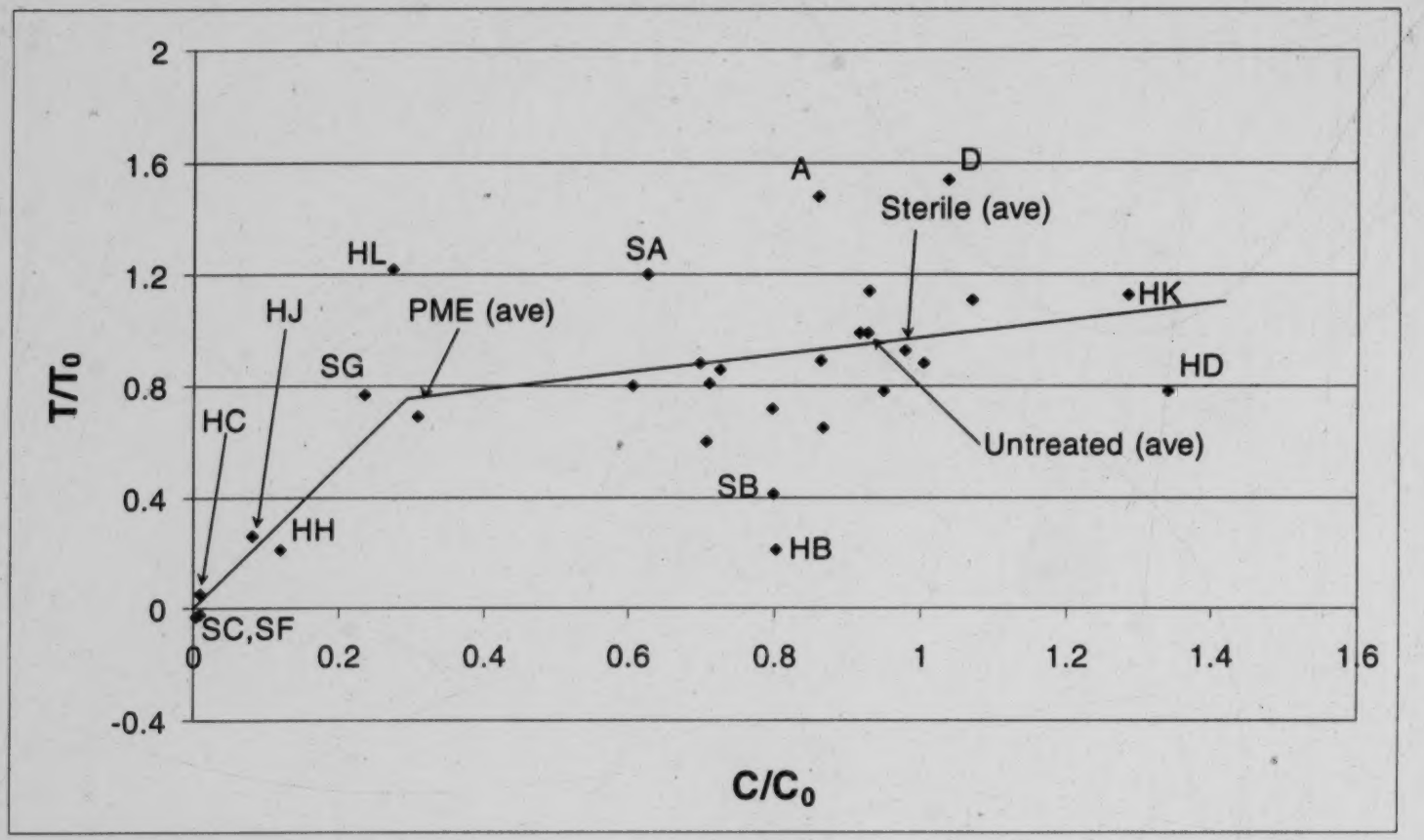

Figure 29: Correlation between C/C_0 and T/T_0

C_0 is the initial concentration of particles sized 2 to 4 µm in diameter, C is the concentration of particles sized 2 to 4 µm after 4 d, T_0 is the initial turbidity reading and T is the turbidity reading after 4 d.

All of A, B, C, D and I were isolates from the Athabasca River water (I), or a 1:1 mixture of Weldwood effluent and Athabasca River water (A, B, C and D). Since these sources were not available during the flocculation experiments, these isolates were re-introduced into a Weyerhaeuser effluent and RW mixture. All of these isolates did grow, but, as was discussed, none of them induced flocculation. A, B and I were characterized as possessing mild flocculation abilites and C and D had no flocculation abilites. Bacteria may be accustomed to a particular environment. If any of these were re-introduced into their native effluent (or one more similar to it), the results may have been different.

From the flocculation experiments, it is obvious that some of the isolates possess flocculating abilities. However this is largely dependent on their physical, chemical and biological environment. The effect of their physical/chemical environment has been discussed. Considering their biological environment, it is possible that some of the species may interact together. Each isolate was tested individually, however interactions between species may be significant. These may help or hinder the flocculation process. The PME control partially examined this. It was grouped with those isolates with C/C_0 (2 to 4 μm) < 0.4, however it did not produce the lowest C/C_0 value. There may not have been sufficient substratum for the increased numbers of bacteria, so more remained in solution. If this was the case, it would have increased the final concentration of particles 2 to 4 μm in diameter. Other complex interactions could also be occurring.

Those isolates that demonstrated enhanced flocculation abilities were HC, HH, HJ, HL, SC, SF and SG. Combining these results with the results of the Biolog identification, a hypothesis of the actual flocculating species can be made. HH and HJ were both positively identified as belonging to the *Pseudomonas* genus, HJ as *P. echinoides* and HH and *Pseudomonas* Group 2 (*Burkholderia*-like). If this is true, then SE, SH, SJ and SN may exhibit flocculation abilites as well, as they were also identified as belonging to the *Pseudomonas* genus. Flocculation abilities may be species specific, however the C/C_0 and T/T_0 results of the only run for these isolates (SE, SH, SJ and SN) is encompassed by the range of all runs of HH and HJ. HC and SF were both classified a enteric, HC likely belonging to the *Enterobacter* genus. SF remains unidentified, as all possible IDs were non-enteric species. It could possibly be an *Aeromonas* species. SG was positively identified as *Bordetella bronchiseptica*, however it is doubtful whether

this pathogen would be abundant in pulp mill effluent or river water. SC was positively identified as *Comamonas testosteroni* and HL remains unidentified (possibly a *Pseudomonas* species).

4.5 Observation of Floc Structure

The correlative microscopy did not proceed as well as imagined. Photographs with all three microscopes were obtained, however the procedure was not smooth and different samples had to be used. There were no major problems with the COM or the TEM. However, the SCLM produced many problems. The staining time was not specified by Droppo et. al. (1996a, 1996b). Therefore various times were tested, ranging from 30 s to 10 h (with a more dilute dye). The optimum was found to be from 5 to 10 min, with a concentrated dye (FITC, 2 mg/mL). However, even within this time frame, the dye would bind to the agar, producing a whole specimen that fluoresced and could not be examined by the human eye with the fluorescent microscope. Washing did not remove enough dye. Occasionally, the dye would not bind to the bacteria, so only the agar would fluoresce. Thus the results shown below are from different samples, the COM photographs from a July sample and the TEM and SCLM photographs from the May sample.

The COM photograph revealed a dense organic matrix of flocculated material (see Figures 30 and 31). Some thin fibers were visible, potentially being of bacterial origin. Visible under the microscope, but not so in the photographs, were many protozoans, actively swimming between the floc particles. Since protozoans rely on bacteria as a food source (Tortora et al., 1992), the bacteria are likely present as well, in the floc particles. In this particular sample, the bacteria did not fluoresce after staining and the TEM was not used, so the presence of bacteria can only be assumed.

Figure 30: Image Taken by COM, of 1:1 Weyerhaeuser Mixture (sampled in July 1999)

Figure 31: Image Taken by COM, of 1:1 Weyerhaeuser Mixture (sampled in July 1999)

The TEM confirmed the presence of bacteria. Individual bacteria were observed; approximately 0.9 to 1.5 μm in length and 0.27 to 0.36 μm in width. Of the bacteria photographed, all appeared rod-shaped. Fuzzy edges were present on some (see Figure 32). This could be a result of the photography, but other structures had definite edges. Thus, it could be the presence of extra-cellular polymeric material, in the form of a capsule. There was no network of long fibrils attached to the individual bacteria, as might be expected. Only one or two "threads" seemed to be attached to the bacteria. The long fibrils may have been sheared from the bacteria during sample processing, or have been released into the surrounding medium by the bacteria (such as the slime layer described by Geesey (1982)), or may not be of bacterial origin at all.

Figure 32: Image Taken by TEM, of bacteria and fibrils.

The bacterium in the bottom left hand corner shows fuzzy edges, representative of EPS.

The second TEM photograph shows a bacterium-bacterium association (see Figure 33). The bacteria are rod-shaped and about 0.8 μm long and 0.4 μm wide. There doesn't appear to be any EPS present, but it could have been removed during sample processing and preparation.

Figure 33: Image Taken by TEM, of a bacteria-bacteria association

It had been hoped that the SCLM would give a constructed 3-D image of the floc. One image was obtained, but it is very difficult to determine the individual structures (see Figure 34). The bacteria did stain in this photograph, so their abundance can be seen easily. Some areas did fluoresce greatly, perhaps because of an abundance of organic matter. However, the dye did not seem to be consistent in what it attached to, so it is difficult to determine for certain.

Figure 34: Constructed Image from SCLM

The bar in the bottom left corner is 10 μm in length

5.0 Limitations

This study was performed in a laboratory setting and many limitations exist in its extrapolation to a field setting. However this stage is required for better understanding of phenomenon in a controlled setting. Firstly, the bacteria species were isolated on prepared media. It is of general consensus among researchers that the majority of environmental bacteria do not grow on these prepared media. So the abundant species that were isolated, may not actually be abundant in the effluent or river water.

The flocculation study used autoclaved effluent and river water, since it was the most convenient method of obtaining sterile effluent. However, this likely changed the physical and chemical properties of both. Bacteria that usually thrive in PME may not do so in the autoclaved effluent, and conversely, some species may prefer the autoclaved PME and RW combination. Also it has been shown that the production of EPS is dependent on the environment (Flemming, 1993; White, 1995). Bacteria that usually produce large amount of EPS may not do so in this autoclaved solution and the reverse may be true as well. Since the production of EPS could aid in the flocculating abilities of bacteria, this has a significant impact on the results of this study.

Finally, the 2-L Erlenmeyer flask used in this experiment, with mixing provided by a stir bar, does not represent the turbulent mixing environment found in a river. The dilution effect present in the river is not simulated in the laboratory at all. The laboratory setting would produce more of a concentration effect, as the bacteria will increase in numbers and no dilution is present.

6.0 Conclusions and Recommendations

Bacteria were isolated from a sample of PME, the corresponding RW and a 1:1 mixture of the two. Identification of these isolates was conducted with the Biolog Identification System. Although there seemed to be many inconsistencies with the identification by the Biolog database, some conclusions could be made. Species that were likely present in the mixture were *Aquaspirillum* spp., *Acidovorax* spp., *Pseudomonas* spp., *Enterobacter* spp., *Comamonas* spp., *Brevundimonas* spp., *Flavobacterium* spp. and *Achromobacter* spp. *Pastuerella* spp and *Aeromonas* may have been present as well.

Through a series of flocculation experiments, it was shown that some of these isolates were capable of enhanced flocculation, in the laboratory setting used. However, this characteristic was not consistent and seemed to vary greatly from experiment to experiment. Based on the Biolog identification, the species possibly involved in enhanced flocculation were *Pseudomonas* spp., *Enterobacter* spp and *Comamonas testosteroni*. Some unidentified isolates were also involved.

With this base of knowledge, further work is recommended in this area. In the laboratory setting, a more detailed identification procedure is needed, so the relevant species can be determined with more certainty. This study focussed on mesophilic aerobes, however the river temperature may be as low as 0°C in the winter, with an ice cover, so a study of the psychrotrophs present would be worthwhile. At the same time, attention should be made to any seasonal variation. It was evident in this study that differences existed between the May and July effluents. Sampling should be conducted over an entire year to have a full picture of the annual trends. Characteristics of the effluent should be monitored, since over an entire year they are likely to change as well. Further work could also explore the production of EPS: how it changes with time, its abundance in non-flocculating solutions as compared to flocculating solutions and its role in the flocculation process.

All the previously mentioned studies can be completed at the laboratory scale. A final step would involve the determination of what actually happens in the river. Many limitations exist for this study, as were discussed, and to extrapolate the laboratory results into the river setting would greatly increase the understanding surrounding this

phenomenon. The rivers involved may be oligotrophic environments, which could produce "stickier" bacteria. This may affect their flocculation abilities. The mixing environment may be too turbulent for the formation of fragile, biological flocs. Or, 4 d after discharge, the time required for biological flocculation to occur, the effluent and its components may be too dilute for any type of reaction.

7.0 References

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APPENDIX

APPENDIX A:
List of Ingredients in Prepared Media

Table A 1: Quantity of Ingredients in R2A (1L)

Ingredient	Quantity (g)
Bacto Yeast Extract	0.5
Bacto Proteose Peptone No. 3	0.5
Bacto Casamino Acids	0.5
Bacto dextrose	0.5
Soluble starch	0.5
Sodium pyruvate	0.3
Potassium phosphate, dibasic	0.3
Magnesium sulfate	0.05
Bacto Agar	15

Table A 2: Quantity of Ingredients in Nutrient Broth (1L)

Ingredient	Quantity (g)
Bacto Beef extract	3
Bacto Peptone	5

Table A 3: Quantity of Ingredients in Standard Plate Count Broth (1L)

Ingredient	Quantity (g)
Bacto Tryptone	5
Bacto Yeast Extract	2.5
Bacto Dextrose (Glucose)	1
Bacto Agar (in solid media only)	15

Table A 4: Quantity of Ingredients in Triple-Sugar-Iron Media, prepared as a slant, with a deep butt

Ingredient	Quantity (%)
Peptone	2
Glucose	0.1
Lactose	1
Sucrose	1
Sodium Chloride	0.5
Ferrous NH_4SO_4	0.02
Sodium thiosulphate	0.02 to 0.03
Phenol red	0.0025
Agar	1.5

APPENDIX B:
Procedures for Characterization Tests

I Procedure for Gram Stain:

1. Heat fix smear of bacteria onto microscope slide.
2. Cover with a basic triphenylmethane dye (crystal violet was used) for about 1 min.
3. Rinse briefly under running water.
4. Cover with iodine, a mordant, for 1 min.
5. Rinse under running water.
6. Wash with ethanol (or an alcohol-acetone solution). Allow solvent to run over tilted slide until dye no longer runs freely from the smear (1-3 s).
7. Rinse immediately under running water.
8. Cover smear with counterstain (safranin) for 1 min.
9. Rinse with running water.
10. Examine slide under microscope. Gram positive bacteria will retain crystal violet dye during wash with ethanol, and appear purple. Gram negative bacteria won't retain crystal violet dye, but will pick up safranin and appear pink.

II Procedure for oxidase test:

1. Grow isolates on solid medium (BAP or R2A were used).
2. Dip sterile swab into Kovács' oxidase reagent (a 1% aqueous solution of tetramethyl-*p*-phenylenediamine dihydrochloride).
3. Touch swab to isolated colony on solid medium and examine swab.
4. If cytochrome c is present, the swab containing the oxidase reagent will be oxidized and turn purple within 10 s. If cytochrome c is not present, the swab will remain its natural colour.

III Procedure for catalase test:

1. Add one drop of hydrogen peroxide (H_2O_2) to a bacterial colony.
2. If the isolate contains catalase (an enzyme that catalyses the decomposition of H_2O_2 to oxygen gas and water), bubbles of gas will appear immediately or within a few seconds, signifying a positive test. If catalase is not present (a negative test), no bubbles will appear.

NOTE: If the bacterial colonies are grown on a blood-containing medium, care must be taken to ensure that the H_2O_2 does not come into contact with the medium: the red blood cells contain catalase and may give rise to a false positive result.

IV Procedure for Triple-sugar-iron (TSI) test:

1. Grow colonies on solid medium (BAP or R2A used).
2. Pick an isolated colony with a stab.
3. Inoculate TSI slant on surface and stab-inoculate deep into the butt.
4. Incubate overnight.
5. Examine slant for:
   - acid/alkaline reaction at the aerobic surface (a yellow colour signifies an acid reaction and a red colour signifies an alkaline reaction).
   - acid/alkaline reaction in the anaerobic butt
   - H_2 or CO_2 production, indicated by a bubble in the bottom of the test tube, or a split in the butt.
   - H_2S production, indicated by blackening of the medium, as a result of ferrous sulphide production.

APPENDIX C:
Map of Biolog GN2-MicroPlate™

Table C 1: Map of Biolog GN2 MicroPlate™ (adapted from Biolog Manual, 1999)

A1 water	A2 α-cyclodextrin	A3 dextrin	A4 glycogen	A5 tween 40	A6 tween 80	A7 N-acetyl-D-galactosamine	A8 N-acetyl-D-glucosamine	A9 adonitol	A10 L-arabinose	A11 D-arabitol	A12 cellobiose
B1 i-erythritol	B2 D-fructose	B3 L-fucose	B4 D-galactose	B5 gentiobiose	B6 α-D-glucose	B7 m-inositol	B8 α-D-lactose	B9 lactulose	B10 maltose	B11 D-mannitol	B12 D-mannose
C1 D-melibiose	C2 β-methyl D-glucoside	C3 D-psicose	C4 D-raffinose	C5 L-rhamnose	C6 D-sorbitol	C7 sucrose	C8 D-trehalose	C9 turanose	C10 xylitol	C11 methyl pyruvate	C12 mono-methyl succinate
D1 acetic acid	D2 cis-aconitic acid	D3 citric acid	D4 formic acid	D5 D-galactonic acid lactone	D6 galacturonic acid	D7 D-gluconic acid	D8 D-glucosaminic acid	D9 D-glucuronic acid	D10 α-hydroxybutyric acid	D11 β-hydroxybutyric acid	D12 γ-hydroxybutyric acid
E1 p-hydroxy phenylacetic acid	E2 itaconic acid	E3 α-keto butyric acid	E4 α-keto glutaric acid	E5 α-keto valeric acid	E6 D,L-lactic acid	E7 malonic acid	E8 propionic acid	E9 quinic acid	E10 D-saccharic acid	E11 sebacic acid	E12 succinic acid
F1 bromo succinic acid	F2 succinamic acid	F3 glucuronamide	F4 alaninamide	F5 D-alanine	F6 L-alanine	F7 L-alanyl-glycine	F8 L-asparagine	F9 L-aspartic acid	F10 L-glutamic acid	F11 glycyl-L-aspartic acid	F12 glycyl-L-glutamic acid
G1 L-histidine	G2 hydroxy L-proline	G3 L-leucine	G4 L-ornithine	G5 L-phenylalanine	G6 L-proline	G7 L-pyroglutamic acid	G8 D-serine	G9 L-serine	G10 L-threonine	G11 D,L-carnitine	G12 γ-amino butyric acid
H1 urocanic acid	H2 inosine	H3 uridine	H4 thymidine	H5 phenyl ethylamine	H6 putrescine	H7 2-amino ethanol	H8 2,3-butanediol	H9 glycerol	H10 D,L-α-glycerol phosphate	H11 glucose-1-phosphate	H12 glucose-6-phosphate

APPENDIX D:
Fingerprint and List of Species for Each Isolate

Table D 1: Fingerprint for A

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	-	-	-	-	+	+	+	-
B	-	+	-	+	-	+	-	-	-	-	+	-
C	-	-	-	-	-	+	-	-	-	+	+	+
D	+	+	+	+	-	-	-	-	-	+	+	-
E	-	-	-	+	-	+	-	+	-	-	-	+
F	+	-	-	+	-	/	-	-	-	-	-	-
G	-	+	-	-	-	+	-	-	-	-	+	/
H	-	-	-	-	-	-	/	/	+	-	-	-

Table D 2: List of Top Ten Species for A

	Species	Probability	SIM	Type
1	*Pseudomonas fluorescens* Biotype G	-	0.20	GN-NENT
2	*Ancylobacter aquaticus*	-	0.14	GN-NENT
3	*Pseudomonas mucidolens*	-	0.06	GN-NENT
4	*Achromobacter cholinophagum*	-	0.02	GN-NENT
5	*Agrobacterium* like-cystic fibrosis	-	0.01	GN-NENT
6	*Enterobacter nimipressuralis*	-	0.00	GN-NENT
7	*Pseudomonas taetrolens*	-	0.00	GN-NENT
8	*Pseudomonas fluorescens*	-	0.00	GN-NENT
9	*Roseomonas fauriae*	-	0.00	GN-NENT
10	*Pseudomonas syringae pv delphinii*	-	0.00	GN-NENT

Table D 3: Fingerprint for B

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	/	-	-	-	-	-	-	-	-	-
B	/	+	-	-	-	+	-	-	-	-	-	-
C	-	/	+	-	-	-	-	-	/	-	+	+
D	+	/	-	+	-	-	-	-	-	+	+	-
E	+	-	+	/	/	+	-	+	-	-	+	+
F	+	+	-	-	-	+	-	-	/	+	-	-
G	-	-	-	-	-	+	-	-	-	-	-	-
H	-	-	-	-	-	-	-	+	-	-	-	-

Table D 4: List of Top Ten Species for B

	Species	Probability	SIM	Type
1	***Roseomonas genomospecies 6***	98	0.71	GN-NENT
2	*Roseomonas fauriae*	2	0.01	GN-NENT
3	*Pseudomonas fluorescens biotype G*	0	0.00	GN-NENT
4	*Pseudomonas aurantiaca*	0	0.00	GN-NENT
5	*Acidovorax facilis*	0	0.00	GN-NENT
6	*Gilardi unnamed rod group 1*	0	0.00	GN-NENT
7	*Acidovorax delafieldii*	0	0.00	GN-NENT
8	*Alcaligenes denitrificans*	0	0.00	GN-NENT
9	*Comamonas terrigena*	0	0.00	GN-NENT
10	*Oligella urethralis*	0	0.00	GN-NENT

Table D 5: Fingerprint for C

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	-	-	-	-	+	+	+	-
B	-	-	-	+	-	+	-	-	-	-	+	-
C	-	-	-	-	-	+	-	-	-	+	+	/
D	+	-	-	+	-	-	-	-	-	+	+	-
E	-	-	-	-	-	+	-	+	-	-	-	-
F	-	-	-	+	-	+	-	-	-	-	-	-
G	-	/	-	-	-	+	-	-	-	-	-	/
H	-	-	-	-	-	-	-	-	+	-	-	-

Table D 6: List of Top Ten Species for C

	Species	Probability	SIM	Type
1	*Pseudomonas fluorescens biotype G*	-	0.20	GN-NENT
2	*Ancyclobacter aquaticus*	-	0.14	GN-NENT
3	*Pseudomonas mucidolens*	-	0.06	GN-NENT
4	*Achromobacter cholinophagum*	-	0.02	GN-NENT
5	*Agrobacterium like-cystic fibrosis*	-	0.01	GN-NENT
6	*Enterobacter nimipressuralis*	-	0.00	GN-ENT
7	*Pseudomonas taetrolens*	-	0.00	GN-NENT
8	*Pseudomonas fluorescens*	-	0.00	GN-NENT
9	*Roseomonas fauriae*	-	0.00	GN-NENT
10	*Pseudomonas syringae PV delphinii*	-	0.00	GN-NENT

Table D 7: Fingerprint for D

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	-	-	-	-	+	+	+	-
B	-	+	-	+	-	+	-	-	-	-	+	-
C	-	-	-	-	-	+	-	-	-	+	+	+
D	+	-	-	+	-	-	-	-	-	/	+	-
E	-	-	-	-	-	+	-	+	-	-	-	+
F	+	-	-	+	-	/	-	-	-	-	-	-
G	-	-	-	-	-	/	-	-	-	-	/	-
H	-	-	-	-	-	-	-	-	-	-	-	-

Table D 8: List of Top Ten Species for D

	Species	Probability	SIM	Type
1	*Agrobacterium* like-cystic fibrosis	-	0.48	GN-NENT
2	*Achromobacter cholinophagum*	-	0.05	GN-NENT
3	*Pseudomonas fluorescens* biotype G	-	0.00	GN-NENT
4	*Agrobacterium vitis* (biovar 3)	-	0.00	GN-NENT
5	*Pseudomonas mucidolens*	-	0.00	GN-NENT
6	CDC Group EO-2	-	0.00	GN-NENT
7	*Actinobacillus rossii*	-	0.00	GN-FAS O
8	*Pseudomonas aurantiaca*	-	0.00	GN-NENT
9	*Roseomonas fauriae*	-	0.00	GN-NENT
10	*Pseudomonas syringae* PV *syringae*	-	0.00	GN-NENT

Table D 9: Fingerprint for I

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	/	-	/	/	-	-	-	-	-	-
B	-	+	-	-	-	+	-	-	/	+	-	+
C	-	-	+	-	-	-	+	-	+	-	-	/
D	/	-	-	-	-	-	-	-	-	-	-	-
E	-	-	/	-	-	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	+	+	-	-	-	-	-	-	-	-

Table D 10: List of Top Ten Species for I

	Species	Probability	SIM	Type
1	***Pasteurella pneumotropica***	97	0.88	GN-NENT
2	***Haemophilus segnis***	1	0.01	GN-NENT
3	***Escherichia coli*** 0157:H7	1	0.01	GN-NENT
4	***Cardiobacterium hominis***	1	0.00	GN-NENT
5	***Pasteurella trehalosi***	0	0.00	GN-NENT
6	***Photobacterium logei***	0	0.00	GN-NENT
7	***Haemophilus parainfluenzae***	0	0.00	GN-NENT
8	***Flavobacterium marinotypicum***	0	0.00	GN-NENT
9	***Aeromonas salmonicida SS achromogenes***	0	0.00	GN-NENT
10	***Haemophillus paragallinarum***	0	0.00	GN-NENT

Table D 11: Fingerprint for HB

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	/	/	-	-	-	-	-	-	-	-
B	-	+	-	-	-	+	-	-	-	+	-	-
C	-	-	-	-	-	-	-	-	-	-	-	+
D	-	-	-	-	-	-	-	-	-	+	+	-
E	-	-	+	-	-	+	-	-	-	-	-	-
F	-	-	-	-	-	-	-	/	+	+	-	-
G	-	-	-	-	-	+	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	+	-	-	-

Table D 12: List of Top Ten Species for HB

	Species	Probability	SIM	Type
1	***Achromobacter cholinophagum***	92	0.65	GN-NENT
2	***Pseudomonas syringae*** PV ***delphinii***	5	0.03	GN-NENT
3	***Neisseria lactamica***	1	0.01	GN-FAS O
4	***Neisseria mucosa***	1	0.01	GN-FAS O
5	***Neisseria subflava***	0	0.00	GN-FAS O
6	***Neisseria perflava***	0	0.00	GN-FAS O
7	***Ochrobactrum anthropi***	0	0.00	GN-NENT
8	***Neisseria flava***	0	0.00	GN-FAS O
9	***Flavobacterium ferrugineum***	0	0.00	GN-NENT
10	***Pseudomonas fluorescens*** biotype G	0	0.00	GN-NENT

Table D 13: Fingerprint for HC

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	+	+	-	-	+	+	-	+	/	+
B	-	+	/	+	+	+	+	-	-	+	+	+
C	-	+	+	-	-	+	+	+	+	-	+	-
D	+	+	+	-	+	+	+	-	+	-	-	-
E	+	-	-	-	-	+	-	-	-	-	-	/
F	+	/	-	+	+	+	+	+	+	+	+	+
G	-	-	-	-	-	-	-	+	+	+	-	-
H	-	+	+	+	-	-	-	-	+	+	+	+

Table D 14: List of Top Ten Species for HC

	Species	Probability	SIM	Type
1	*Enterobacter asburiae*	-	0.69	GN-ENT
2	*Anterobacter cancerogenus/taylorae*	-	0.00	GN-ENT
3	*Enterobacter hormaechei*	-	0.00	GN-ENT
4	*Enterobacter aerogenes (Klb. mobilus)*	-	0.00	GN-ENT
5	*Klebsiella pneumoniae* ss *pneumoniae*	-	0.00	GN-ENT
6	*Kluyvera ascorbata*	-	0.00	GN-ENT
7	*Aeromonas sobria* DNA group 7	-	0.00	GN-NENT
8	*Enterobacter cloacae*	-	0.00	GN-ENT
9	*Cedecea neteri*	-	0.00	GN-ENT
10	*Klebsiella terrigena*	-	0.00	GN-ENT

Table D 15: Fingerprint for HD

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	+	+	/	+	+	-	-	-	+	-	/
B	/	+	-	+	-	+	-	-	-	/	-	+
C	+	-	+	-	-	-	-	-	+	-	+	+
D	+	+	-	+	-	-	+	-	-	+	+	+
E	+	/	+	+	+	+	-	+	+	+	+	+
F	+	+	-	+	+	+	/	+	+	+	-	+
G	-	-	+	-	+	+	-	-	+	+	-	+
H	+	/	-	-	-	-	-	-	/	-	-	-

Table D 16: List of Top Ten Species for HD

	Species	Probability	SIM	Type
1	***Acidovorax delafieldii***	100	0.69	GN-NENT
2	*Comamonas acidovorans*	0	0.00	GN-NENT
3	*Acinetobacter calcoaceticus*/genospecies 1	0	0.00	GN-NENT
4	*Pseudomonas aurantiaca*	0	0.00	GN-NENT
5	*Ralstonia eutropha*	0	0.00	GN-NENT
6	*Pseudomonas bathycetes*	0	0.00	GN-NENT
7	*Ralstonia pickettii*	0	0.00	GN-NENT
8	*Comamonas testosteroni*	0	0.00	GN-NENT
9	*Pseudomonas putida* biotype B	0	0.00	GN-NENT
10	*Alcaligenes xylosoxydans*	0	0.00	GN-NENT

Table D 17: Fingerprint for HE

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	/	/	+	+	-	-	-	+	-	-
B	-	+	-	-	-	+	-	-	-	-	-	-
C	-	-	+	-	-	-	-	-	-	-	+	+
D	+	-	/	/	-	-	+	-	-	+	+	-
E	+	-	/	+	-	+	-	+	+	-	+	+
F	+	+	-	+	+	+	-	+	+	+	-	/
G	+	-	+	-	+	+	-	-	-	-	-	+
H	+	-	-	-	-	-	-	-	+	-	-	-

Table D 18: List of Top Ten Species for HE

	Species	Probability	SIM	Type
1	***Acidovorax delafieldii***	85	0.50	GN-NENT
2	*Acinetobacter calcoaceticus*/genospecies 1	12	0.07	GN-NENT
3	*Comamonas acidovorans*	2	0.01	GN-NENT
4	*Pseudomonas fluorescens* biotype G	0	0.00	GN-NENT
5	*Ralstonia eutropha*	0	0.00	GN-NENT
6	*Pseudomonas fluorescens* biotype C	0	0.00	GN-NENT
7	*Pseudomonas fuscovaginae*	0	0.00	GN-NENT
8	*Pseudomonas chlororaphis* (*flour.* biotype D)	0	0.00	GN-NENT
9	*Acidovorax facilis*	0	0.00	GN-NENT
10	*Pseudomonas asplenii*	0	0.00	GN-NENT

Table D 19: Fingerprint for HF

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	+	+	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	+	-
D	+	-	-	+	-	-	-	-	-	+	+	-
E	-	-	+	-	-	+	-	+	-	-	-	-
F	-	-	-	+	/	/	-	+	+	+	-	-
G	-	-	-	-	-	-	-	-	+	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

Table D 20: List of Top Ten Species for HF

	Species	Probability	SIM	Type
1	***Aquaspirillum metamorphum***	87	0.57	GN-NENT
2	*Aquaspirillum dispar*	8	0.05	GN-NENT
3	*Acinetobacter johnsonii*/genospecies 7	3	0.02	GN-NENT
4	*Comamonas terrigena*	0	0.00	GN-NENT
5	*Burkeholderia cepacia*	0	0.00	GN-NENT
6	*Pseudomonas fluorescens* biotype G	0	0.00	GN-NENT
7	*Variovorax paradoxus*	0	0.00	GN-NENT
8	*Aquaspirillum putridiconchyliumi*	0	0.00	GN-NENT
9	*Acidovorax delfafieldii*	0	0.00	GN-NENT
10	*Francisella philomiragia*	0	0.00	GN-ENT

Table D 21: Fingerprint for HG

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	+	+	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	-	-
D	/	-	-	-	-	-	-	-	-	-	+	-
E	-	-	/	+	+	-	-	/	-	-	-	-
F	-	/	-	-	-	-	-	-	-	/	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

Table D 22: List of Top Ten Species for HG

	Species	Probability	SIM	Type
1	*Sphingomonas macrogoltabidus*	-	0.27	GN-NENT
2	*Acinetobacter radioreesistens*/genospecies 12	-	0.13	GN-NENT
3	*Aeromonas salmonicida* SS *salmonicida*	-	0.13	GN-NENT
4	*Variovorax paradoxus*	-	0.07	GN-NENT
5	*Aquaspirillum peregrinum* SS *integrum*	-	0.06	GN-NENT
6	*Acidovorax delafieldii*	-	0.06	GN-NENT
7	*Oligella ureolytica*	-	0.03	GN-NENT
8	*Pseudomonas stutzeri*	-	0.02	GN-NENT
9	*Psychrobacter immobilis*	-	0.02	GN-NENT
10	*Flavobacterium tirrenicum (chryseobacterium)*	-	0.02	GN-ENT

Table D 23: Fingerprint for HH

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	/	+	+	+	+	-	+	+	+	+	-
B	/	+	+	+	/	+	-	-	-	/	+	+
C	-	/	+	-	-	+	-	/	+	+	+	+
D	+	+	+	+	+	+	+	-	+	+	+	-
E	+	+	+	+	+	+	+	+	+	+	+	+
F	+	+	-	+	+	+	-	+	+	+	-	-
G	/	-	+	-	+	+	+	/	+	+	-	+
H	+	-	-	-	-	-	+	-	+	-	-	-

Table D 24: List of Top Ten Species for HH

	Species	Probability	SIM	Type
1	***Pseudomonas* Group 2 (*Burkholderia*-like)**	95	0.59	GN-NENT
2	*Pseudomonas rubrisubalbicans*	5	0.03	GN-NENT
3	*Pseudomonas floridana (Burkholderia*-like)	0	0.00	GN-NENT
4	*Burkholderia cepacia*	0	0.00	GN-NENT
5	*Pseudomonas huttiensis (Burkholderia*-like)	0	0.00	GN-NENT
6	*Herbaspirillum seropedicae*	0	0.00	GN-NENT
7	*Pseudomonas phenazinium (Burkholderia*-like)	0	0.00	GN-NENT
8	*Pseudomonas glathei (Burkholderia*-like)	0	0.00	GN-NENT
9	*Flavimonas oryzihabitans*	0	0.00	GN-NENT
10	*Burkholderia vietnamiensis*	0	0.00	GN-NENT

Table D 25: Fingerprint for HI

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	/	/	-	-	-	-	-	-	-	-
B	-	/	-	-	-	-	-	-	-	-	-	+
C	-	-	-	-	-	-	/	-	-	-	-	+
D	-	-	-	-	-	-	-	-	-	-	+	-
E	-	-	+	-	-	+	-	-	-	-	-	-
F	-	/	-	-	-	-	-	/	-	/	-	-
G	-	-	-	-	-	/	-	-	/	/	-	-
H	-	-	-	-	-	-	-	-	/	-	-	-

Table D 26: List of Top Ten Species for HI

	Species	Probability	SIM	Type
1	*Aquaspirillum putridiconchylium*	-	0.35	GN-NENT
2	*Roseomonas fauriae*	-	0.12	GN-NENT
3	*Acidovorax facilis*	-	0.09	GN-NENT
4	*Chryseobacterium indoltheticum*	-	0.08	GN-NENT
5	CDC Group EO-2	-	0.08	GN-NENT
6	*Lampropedia hyalina*	-	0.03	GN-NENT
7	*Aquaspirillum peregrinum*	-	0.03	GN-NENT
8	*Agrobacterium* like-cystic fibrosis	-	0.02	GN-NENT
9	*Francisella philomiragia*	-	0.02	GN-ENT
10	*Bordetella parapertussis*	-	0.02	GN-NENT

Table D 27: Fingerprint for HJ

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	/	-	-	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	+	+
D	/	-	-	-	-	-	-	-	-	-	+	-
E	-	-	+	-	+	+	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-
G	-	-	-	-	-	+	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	+	-	-	-

Table D 28: List of Top Ten Species for HJ

	Species	Probability	SIM	Type
1	*Pseudomonas echinoides*	92	0.80	GN-NENT
2	*Hydrogenophaga flava*	5	0.04	GN-NENT
3	*Acidovorax delafieldii*	2	0.02	GN-NENT
4	*Aquaspirillum peregrinum*	0	0.00	GN-NENT
5	*Aquaspirillum metamorphum*	0	0.00	GN-NENT
6	*Comamonas terrigena*	0	0.00	GN-NENT
7	*Pseudomonas carboxydohydrogena (hydrogenophaga)*	0	0.00	GN-NENT
8	*Achromobacter cholinophagum*	0	0.00	GN-NENT
9	*Acidovorax facilis*	0	0.00	GN-NENT
10	*Neisseria flavescens*	0	0.00	GN-FAS O

Table D 29: Fingerprint for HK

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	+	+	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	+	/
D	+	-	-	+	-	-	-	-	-	+	+	-
E	-	-	+	-	-	+	-	+	-	-	-	-
F	-	-	-	+	+	/	-	+	+	+	-	-
G	-	-	-	-	-	-	-	-	+	-	-	-
H	/	-	-	-	-	-	-	-	-	-	-	-

Table D 30: List of Top Ten Species for HK

	Species	Probability	SIM	Type
1	***Aquaspirillum metamorphum***	83	0.53	GN-NENT
2	*Aquaspirillum dispar*	7	0.04	GN-NENT
3	*Burkholderia cepacia*	5	0.03	GN-NENT
4	*Acinetobacter johnsonii*/genospecies 7	4	0.02	GN-NENT
5	*Comamonas terrigena*	1	0.00	GN-NENT
6	*Aquaspirillum putridiconchylium*	0	0.00	GN-NENT
7	*Pseudomonas fluorescens* biotype G	0	0.00	GN-NENT
8	*Acidovorax delafieldii*	0	0.00	GN-NENT
9	*Francisella philomiragia*	0	0.00	GN-ENT
10	*Hydrogenophaga palleronii*	0	0.00	GN-NENT

Table D 31: Fingerprint for HL

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	-	-	-	-	-	-	-	-
B	-	-	-	/	-	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	+	+
D	/	-	-	-	-	-	-	-	-	-	+	-
E	-	-	/	-	+	+	-	+	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	/
G	-	-	-	-	-	/	-	-	/	-	-	-
H	-	-	-	-	-	-	-	-	/	-	-	-

Table D 32: List of Top Ten Species for HL

	Species	Probability	SIM	Type
1	*Lampropedia hyalina*	-	0.49	GN-NENT
2	*Pseudomonas echinoides*	-	0.39	GN-NENT
3	*Acidovorax delafieldii*	-	0.03	GN-NENT
4	*Roseomonas fauriae*	-	0.01	GN-NENT
5	*Aquaspirillum peregrinum*	-	0.00	GN-NENT
6	CDC Group EO-2	-	0.00	GN-NENT
7	*Aquaspirillum putridiconchylium*	-	0.00	GN-NENT
8	*Pseudomonas carboxydohydrogena (hydrogenophaga)*	-	0.00	GN-NENT
9	*Hydrogenophaga flava*	-	0.00	GN-NENT
10	*Moraxella catarrhalis*	-	0.00	GN-FAS O

Table D 33: Fingerprint for SA

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	+	-	+	+	-	/	-	-	-	+
B	-	+	-	/	+	+	-	-	-	+	-	+
C	-	-	-	-	-	-	-	-	-	-	+	-
D	-	-	-	-	-	-	-	-	-	-	+	-
E	-	-	+	-	-	+	-	/	-	-	-	/
F	-	-	-	+	-	+	+	+	/	+	/	+
G	-	-	-	-	-	/	-	-	+	/	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

Table D 34: List of Top Ten Species for SA

	Species	Probability	SIM	Type
1	***Brevundimonas vesicularis***	88	0.50	GN-NENT
2	*Stenotrophomonas maltophilia*	4	0.02	GN-NENT
3	*Achromobacter cholinophagum*	3	0.02	GN-NENT
4	*Vibrio campbelli*	2	0.01	GN-NENT
5	*Janthinobacterium lividum* B	2	0.01	GN-NENT
6	*Flavobacterium hydatis*	1	0.00	GN-NENT
7	*Janthinobacterium lividum* C	0	0.00	GN-NENT
8	*Sphingomonas adhaesiva*	0	0.00	GN-NENT
9	*Aeromonas veronii/sobria* DNA Group 8	0	0.00	GN-NENT
10	*Flavobacterium marinotypicum*	0	0.00	GN-NENT

Table D 35: Fingerprint for SB

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	+	-	-	-	-	-	-	-	-	-
B	-	+	+	+	-	+	-	-	-	+	+	+
C	-	-	+	-	-	-	+	-	+	-	+	+
D	-	-	-	-	-	-	-	-	-	-	-	-
E	+	-	+	-	-	-	-	-	-	-	-	-
F	+	-	-	-	-	-	-	-	+	-	+	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	+	-	-	+	-	-	-

Table D 36: List of Top Ten Species for SB

	Species	Probability	SIM	Type
1	***Flavobacterium marinotypicum***	94	0.53	GN-NENT
2	*Achromobacter cholinophagum*	6	0.03	GN-NENT
3	*Cardiobacterium hominis*	0	0.00	GN-NENT
4	*Yersinia ruckeri*	0	0.00	GN-ENT
5	*Enterobacter nimipressuralis*	0	0.00	GN-ENT
6	*Erwinia carotovora* SS *atroseptica*	0	0.00	GN-ENT
7	*Erwinia chrysanthemi*	0	0.00	GN-ENT
8	*Actinobacillus seminis*	0	0.00	GN-FAS O
9	*Suttonella indologenes*	0	0.00	GN-FAS O
10	*Agrobacterium vitis* (biovar 3)	0	0.00	GN-NENT

Table D 37: Fingerprint for SC

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	+	-	/	+	+	-	-	-	-	-	-
B	-	/	-	-	-	-	-	-	-	-	-	-
C	-	-	-	-	-	/	-	-	-	-	+	+
D	+	+	-	+	/	-	/	-	-	+	+	-
E	-	+	+	+	+	+	/	+	-	-	+	+
F	+	+	-	-	-	-	-	+	+	+	-	-
G	-	-	+	-	-	+	+	-	-	+	-	-
H	-	-	-	-	/	/	/	/	/	/	-	-

Table D 38: List of Top Ten Species for SC

	Species	Probability	SIM	Type
1	***Comamonas testosteroni***	91	0.71	GN-NENT
2	*Comamonas acidovorans*	6	0.05	GN-NENT
3	*Aquaspirillum autotrophicum*	2	0.01	GN-NENT
4	*Comamonas terrigena*	1	0.01	GN-NENT
5	*Acidovorax avenae* SS *citruli*	0	0.00	GN-NENT
6	*Acidovorax delafieldii*	0	0.00	GN-NENT
7	*Bordetella hinzii*	0	0.00	GN-NENT
8	*Pseudomonas fluorescens* biotype C	0	0.00	GN-NENT
9	*Acidovorax temperans*	0	0.00	GN-NENT
10	CDC Group IVC-2 (*Alcaligenes*-like)	0	0.00	GN-NENT

Table D 39: Fingerprint for SE

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	+	+	-	+	-	-	-	-
B	-	+	-	-	-	+	+	-	-	-	+	+
C	-	-	+	-	-	-	+	+	-	-	+	-
D	+	+	+	/	-	-	+	-	-	-	+	-
E	+	-	-	+	+	+	+	+	+	-	-	+
F	+	-	-	-	-	/	/	+	+	+	-	-
G	+	+	+	+	-	+	/	-	+	-	-	+
H	+	+	-	-	-	+	+	/	+	+	-	+

Table D 40: List of top Ten Species for SE

	Species	Probability	SIM	Type
1	*Pseudomonas fiscovaginae*	-	0.40	GN-NENT
2	*Pseudomonas synxantha*	-	0.06	GN-NENT
3	*Pseudomonas chlororaphis (fluor. biotype D)*	-	0.00	GN-NENT
4	*Pseudomonas fluorescens* biotype G	-	0.00	GN-NENT
5	*Pseudomonas aureofaciens (fluor.* biotype E)	-	0.00	GN-NENT
6	*Pseudomonas aeruginosa*	-	0.00	GN-NENT
7	*Pseudomonas aurantiaca*	-	0.00	GN-NENT
8	*Pseudomonas fluorescens* biotype C	-	0.00	GN-NENT
9	*Pseudomonas putida*	-	0.00	GN-NENT
10	*Pseudomonas asplenii*	-	0.00	GN-NENT

Table D 41: Fingerprint for SF

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	+	+	/	-	-	+	-	-	-	-
B	-	+	-	-	-	+	-	-	-	+	-	+
C	-	+	+	-	-	-	+	+	+	-	-	/
D	-	-	-	-	-	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-	/	-	-	-	+
F	-	-	-	-	-	-	-	/	-	-	/	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	/	-	-	+

Table D 42: List of Top Ten Species for SF

	Species	Probability	SIM	Type
1	*Aeromonas eucrenophilia* DNA Group 6	-	0.39	GN-NENT
2	*Vibrio metschnikovii*	-	0.26	GN-NENT
3	*Vibrio parahaemolyticus*	-	0.06	GN-NENT
4	*Aeromonas hydrophila*-like DNA Group 2	-	0.03	GN-NENT
5	*Aeromonas veronii* DNA Group 10	-	0.03	GN-NENT
6	*Cardiobacterium hominis*	-	0.00	GN-NENT
7	*Vibrio harveyi*	-	0.00	GN-NENT
8	*Photobacterium logei*	-	0.00	GN-NENT
9	*Achromobacter cholinophagum*	-	0.00	GN-NENT
10	*Vibrio alginolyticus*	-	0.00	GN-NENT

Table D 43: Fingerprint for SG

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	/	+	+	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-
C	-	/	-	-	-	-	-	-	-	-	+	+
D	+	+	+	+	-	-	-	-	-	+	+	-
E	-	/	+	+	+	+	-	+	-	-	-	+
F	+	/	-	+	+	+	+	+	+	+	-	-
G	/	-	+	+	+	+	/	/	+	+	-	/
H	-	-	-	-	-	+	+	-	-	-	-	-

Table D 44: List of Top Ten Species for SG

	Species	Probability	SIM	Type
1	***Bordetella bronchiseptica***	97	0.72	GN-NENT
2	*Pseudomonas alcaligenes*	1	0.01	GN-NENT
3	*Bordetella hinzii*	1	0.06	GN-NENT
4	*Pseudomonas pseudoalcaligenes*	0	0.00	GN-NENT
5	*Acinetobacter calcoaceticus*/genospecies 1	0	0.00	GN-NENT
6	*Bordetella trematum*	0	0.00	GN-NENT
7	*Pseudomonas fluorescens* biotype C	0	0.00	GN-NENT
8	*Pseudomonas denitrificans*	0	0.00	GN-NENT
9	*Alcaligenes faecalis*	0	0.00	GN-NENT
10	*Bordetella*-like species	0	0.00	GN-NENT

Table D 45: Fingerprint for SH

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	/	+	+	-	-	-	-	-	-
B	/	-	-	-	-	-	-	-	-	-	-	-
C	-	/	-	-	-	-	-	-	-	-	+	+
D	+	+	+	+	-	-	-	-	-	+	/	-
E	-	-	+	+	+	+	-	+	-	-	-	+
F	+	/	-	+	+	+	+	+	+	+	-	-
G	-	-	/	/	-	+	-	-	+	+	-	-
H	-	-	-	-	-	+	+	-	-	-	-	-

Table D 46: List of Top Ten Species for SH

	Species	Probability	SIM	Type
1	*Pseudomonas fluorescens* biotype C	-	0.31	GN-NENT
2	*Pseudomonas pseudoalcaligenees*	-	0.12	GN-NENT
3	*Pseudomonas alcaligenes*	-	0.08	GN-NENT
4	*Bordetella brochiseptica*	-	0.07	GN-NENT
5	*Pseudomonas chlororaphis (fluor.* biotype D)	-	0.03	GN-NENT
6	*Pseudomonas aurantiaca*	-	0.00	GN-NENT
7	*Comomonas terrigena*	-	0.00	GN-NENT
8	*Acinetobacter calcoaceticus*/genospecies 1	-	0.00	GN-NENT
9	*Bordetella hinzii*	-	0.00	GN-NENT
10	*Pseudomonas fulva*	-	0.00	GN-NENT

Table D 47: Fingerprint for SI

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	+	-	-	+	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-
C	-	/	-	-	-	-	-	-	-	-	+	-
D	-	/	-	-	-	-	-	-	-	-	-	-
E	-	-	+	+	+	/	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-
G	-	-	-	-	/	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

Table D 48: List of Top Ten Species for SI

	Species	Probability	SIM	Type
1	*Pasteurella trehalosi*	-	0.22	GN-NENT
2	*Neisseria denitrificans*	-	0.14	GN-FAS O
3	*Neisseria elongata*	-	0.12	GN-FAS O
4	*Alysiella filiformis*	-	0.10	GN-FAS O
5	*Neisseria canis*	-	0.09	GN-FAS O
6	*Neisseria subflava*	-	0.02	GN-FAS O
7	*Neisseria meningitidis*	-	0.02	GN-FAS O
8	*Simonsiella crassa*	-	0.01	GN-FAS O
9	*Neisseria flavescens*	-	0.01	GN-FAS O
10	*Acinetobacter johnsonii*/genospecies 7	-	0.01	GN-NENT

Table D 49: Fingerprint for SJ

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	-	-	-	-	-	-	-	-
B	-	-	-	-	-	+	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	+	+
D	+	-	+	+	+	-	+	-	-	+	+	+
E	/	-	+	+	/	+	-	+	-	-	-	+
F	+	+	-	-	-	-	-	-	-	+	-	-
G	-	-	-	-	-	-	+	-	-	-	-	-
H	-	-	-	-	-	-	-	/	-	-	-	-

Table D 50: List of Top Ten Species for SJ

	Species	Probability	SIM	Type
1	*Pseudomonas aurantiaca*	-	0.33	GN-NENT
2	*Bordetella hinzii*	-	0.11	GN-NENT
3	*Pseudomonas fluorescens biotype C*	-	0.10	GN-NENT
4	*Pseudomonas citronellolis*	-	0.03	GN-NENT
5	*Roseomonas fauriae*	-	0.02	GN-NENT
6	*Aquaspirillum autotrophicum*	-	0.01	GN-NENT
7	*Acidovorax facilis*	-	0.00	GN-NENT
8	*Pseudomonas taetrolens*	-	0.00	GN-NENT
9	*Pseudomonas mucidolens*	-	0.00	GN-NENT
10	*Rosemonas genomospecies 6*	-	0.00	GN-NENT

Table D 51: Fingerprint for SN

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	-	-	-	-	-	-	-	-
B	-	-	-	-	-	+	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	+	+
D	+	/	+	+	+	/	+	-	-	+	+	+
E	/	-	+	+	/	+	-	+	-	-	-	+
F	+	+	-	-	-	-	-	-	-	+	-	-
G	-	-	-	-	-	-	+	-	-	-	-	-
H	-	-	-	-	-	-	-	/	-	-	-	-

Table D 52: List of Top Ten Species for SN

	Species	Probability	SIM	Type
1	*Pseudomonas aurantiaca*	-	0.41	GN-NENT
2	*Bordetella hinzii*	-	0.13	GN-NENT
3	*Pseudomonas fluorescens biotype C*	-	0.12	GN-NENT
4	*Pseudomonas citronellolis*	-	0.00	GN-NENT
5	*Roseomonas fauriae*	-	0.00	GN-NENT
6	*Aquaspirillum autotrophicum*	-	0.00	GN-NENT
7	*Pseudomonas fluorescens biotype G*	-	0.00	GN-NENT
8	*Bordetella parapertussis*	-	0.00	GN-NENT
9	*Alcaligenes denitrficans*	-	0.00	GN-NENT
10	*Acidovorax facilis*	-	0.00	GN-NENT

Table D 53: Fingerprint for *E. coli*

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	+	-	-	-	-	+	-	+	-	-
B	-	+	+	+	-	+	-	+	-	+	+	+
C	-	-	+	-	-	-	-	+	/	-	+	+
D	-	-	-	+	-	-	+	-	/	-	-	-
E	-	-	-	-	-	+	-	-	-	-	-	+
F	+	-	+	/	/	+	/	+	+	-	+	/
G	-	-	-	-	-	-	-	+	+	+	-	-
H	-	+	+	+	-	-	-	-	+	-	+	+

Table D 54: List of Top Ten Species for *E. coli*

	Species	Probability	SIM	Type
1	*Escherichia coli*	-	0.67	GN-ENT
2	*Escherichia coli* 0157:H7	-	0.03	GN-ENT
3	*Salmonella* GP 6 *(indica)*	-	0.02	GN-ENT
4	*Hafnia alvei*	-	0.01	GN-ENT
5	*Citrobacter youngae*	-	0.00	GN-ENT
6	*Shigella sonnei*	-	0.00	GN-ENT
7	*Salmonella* GP 1 *(choleraesuis)*	-	0.00	GN-ENT
8	*Salmonella* GP 3A *(arizonae)*	-	0.00	GN-ENT
9	*Citrobacter braakii*	-	0.00	GN-ENT
10	*Citrobacter freundii*	-	0.00	GN-ENT

APPENDIX E:
Particle Size Distributions

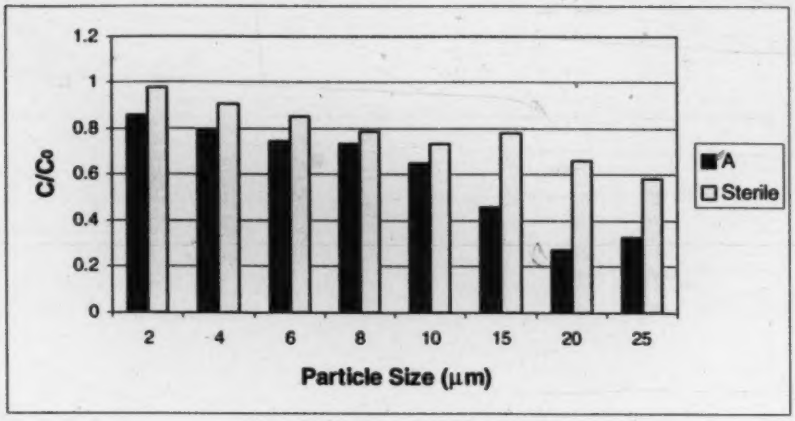

Figure E 1: Change in Particle Size Distribution for A

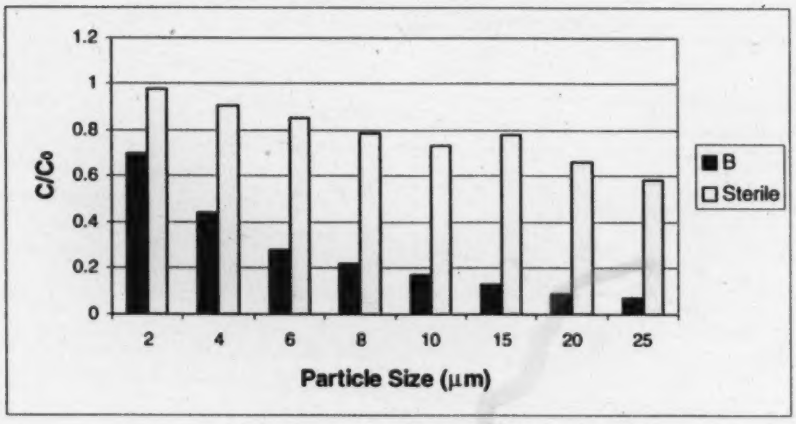

Figure E 2: Change in Particle Size Distribution for B

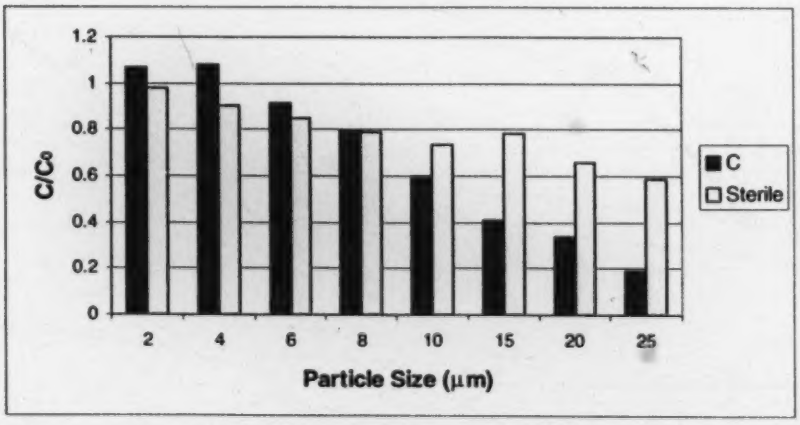

Figure E 3: Change in Particle Size Distribution for C

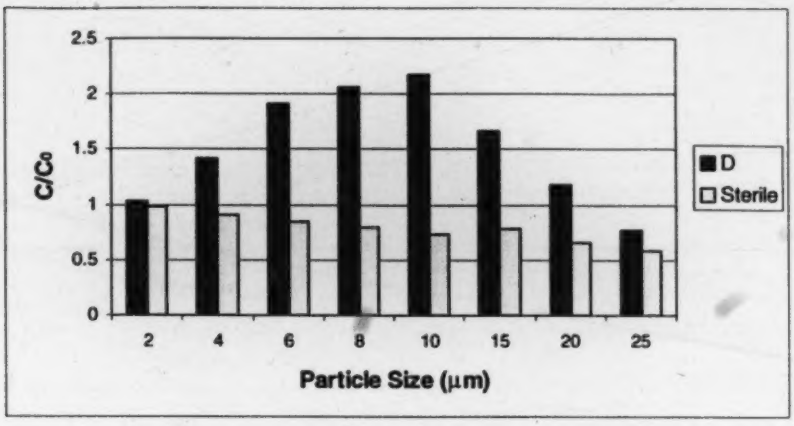

Figure E 4: Change in Particle Size Distribution for D

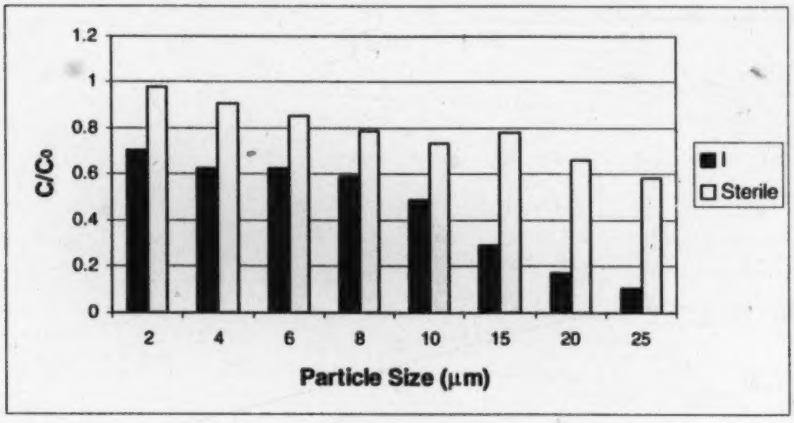

Figure E 5: Change in Particle Size Distribution for I

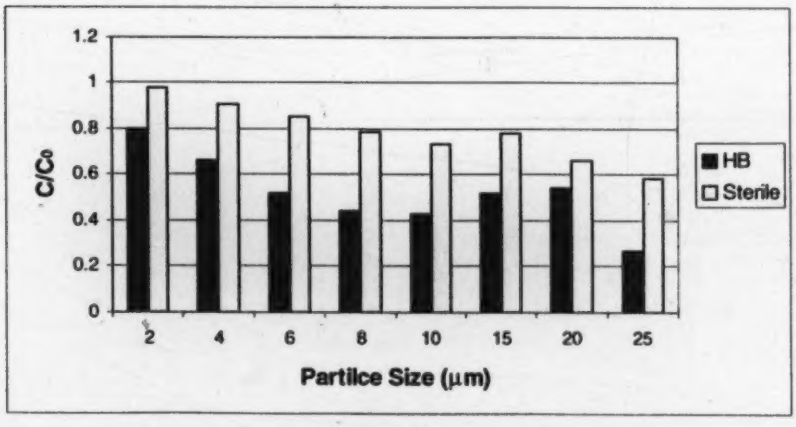

Figure E 6: Change in Particle Size Distribution for HB

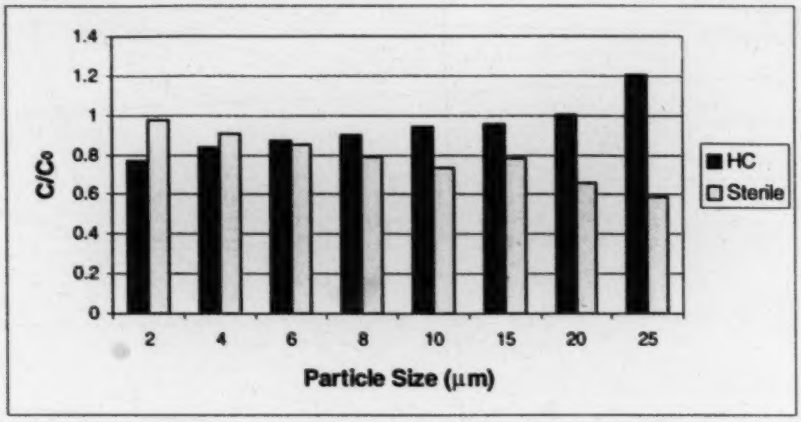

Figure E 7: Change in Particle Size Distribution for HC (average of 4 runs)

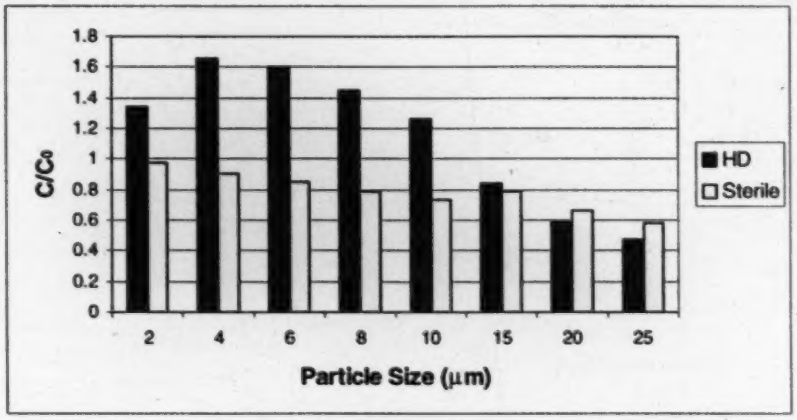

Figure E 8: Change in Particle Size Distribution for HD

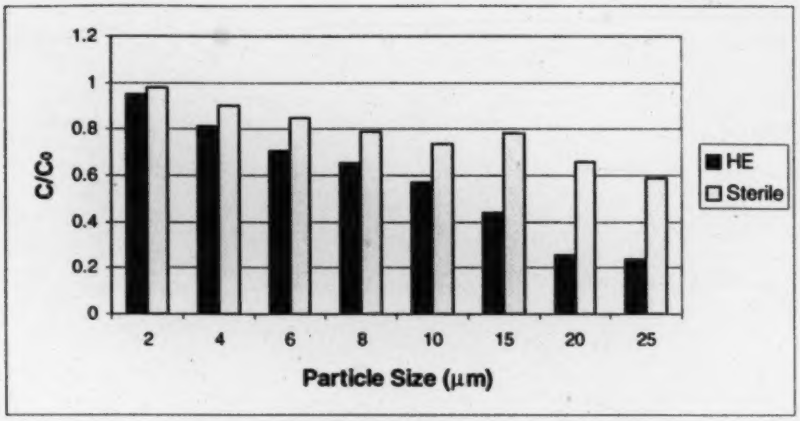

Figure E 9: Change in Particle Size Distribution for HE

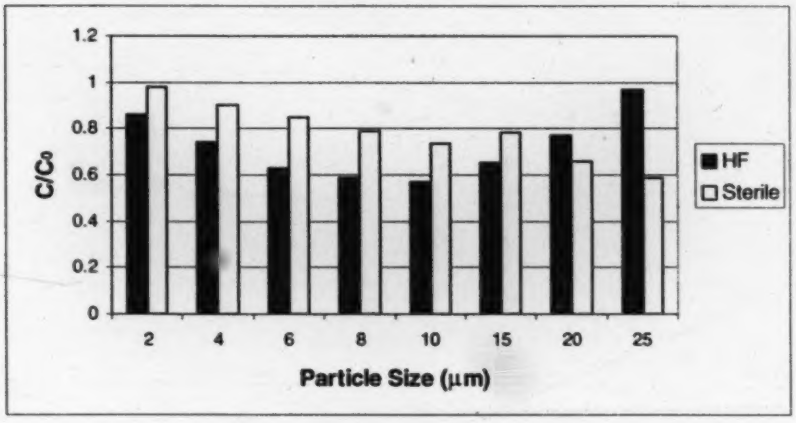

Figure E 10: Change in Particle Size Distribution for HF

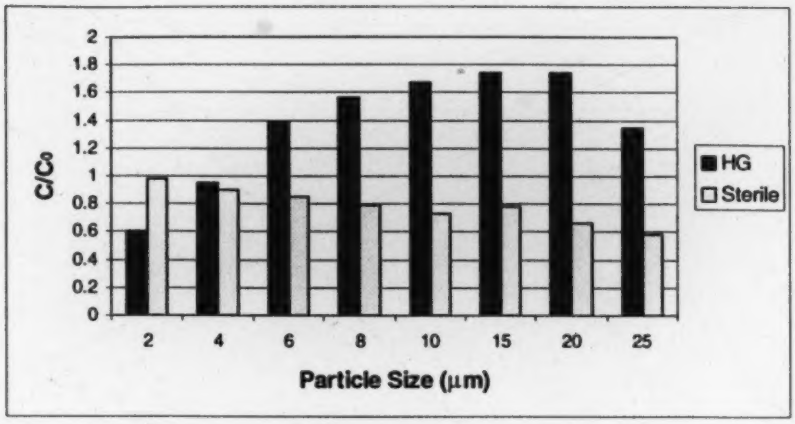

Figure E 11: Change in Particle Size Distribution for HG

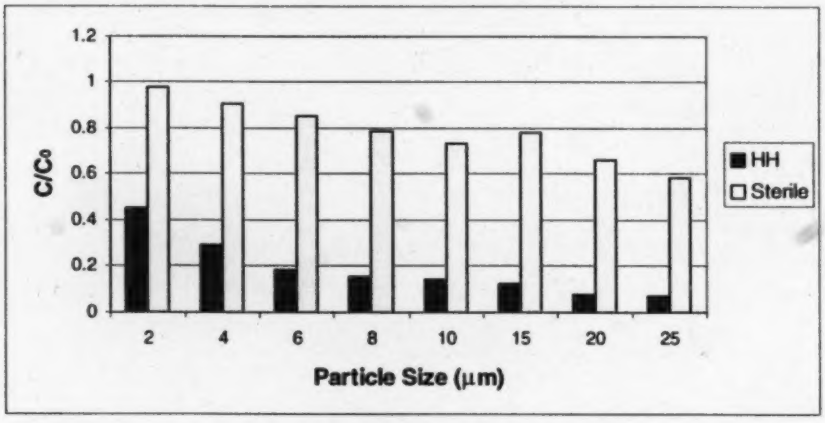

Figure E 12 : Change in Particle Size Distribution for HH (average of 2 runs)

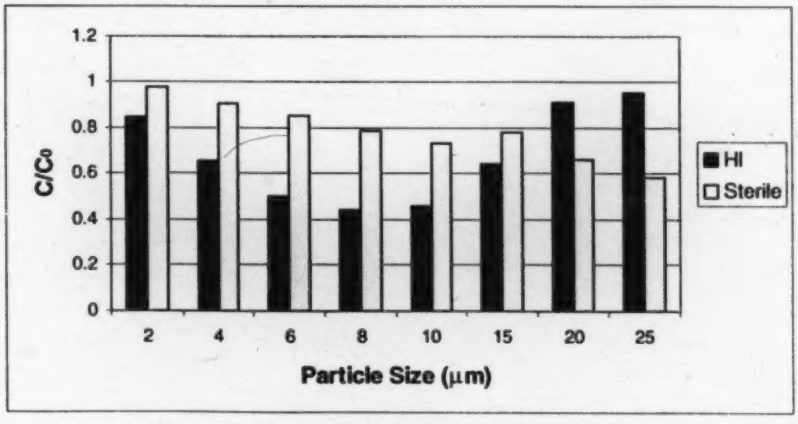

Figure E 13: Change in Particle Size Distribution for HI

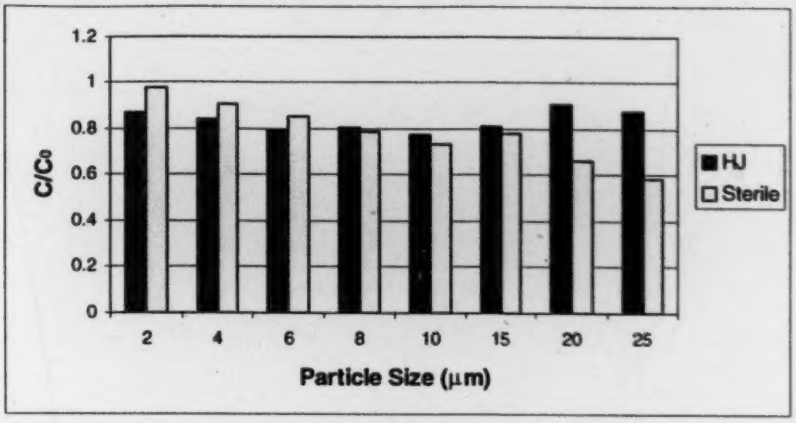

Figure E 14: Change in Particle Size Distribution for HJ (average of 4 runs)

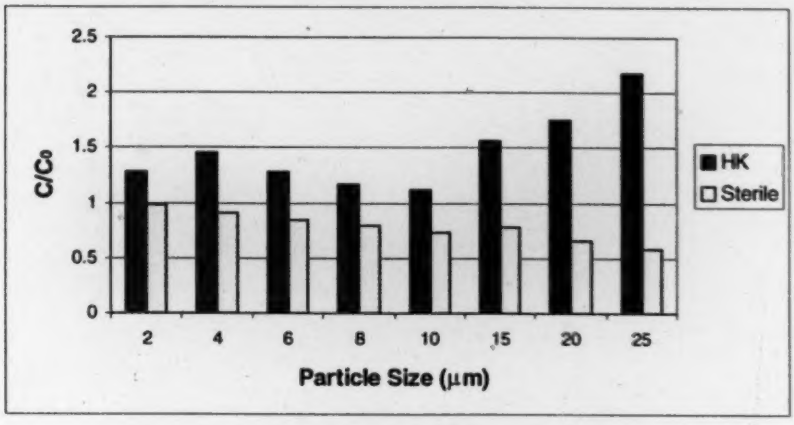

Figure E 15: Change in Particle Size Distribution for HK

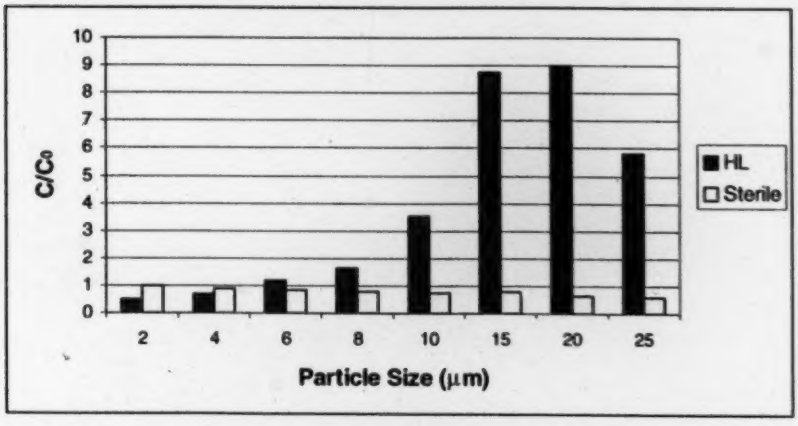

Figure E 16: Change in Particle Size Distribution for HL (average of 3 runs)

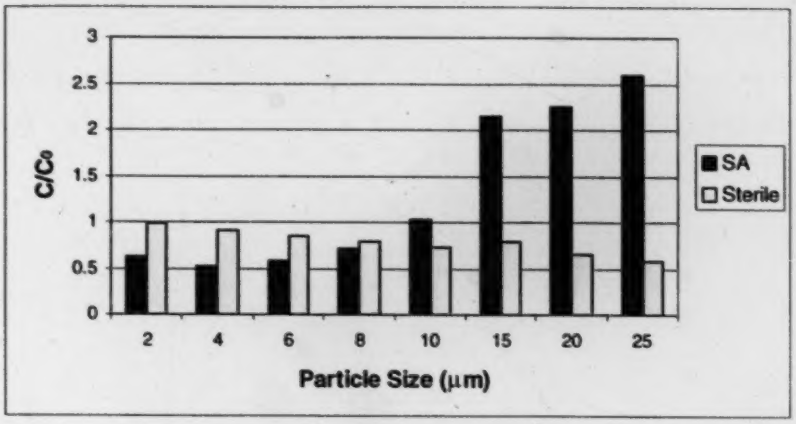

Figure E 17: Change in Particle Size Distribution for SA

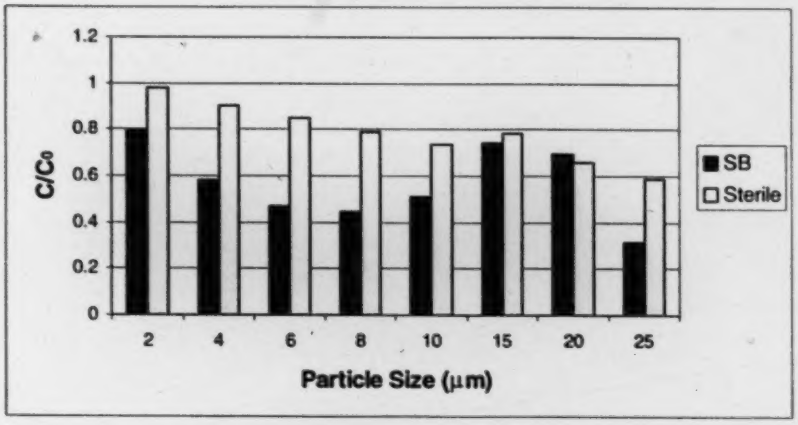

Figure E 18: Change in Particle Size Distribution for SB

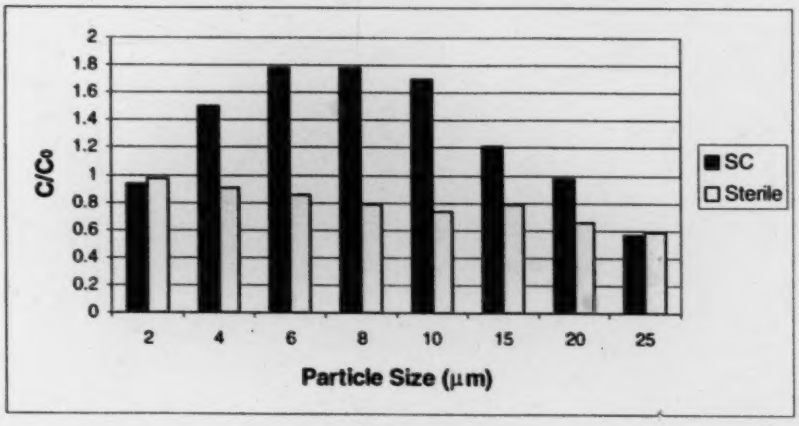

Figure E 19: Change in Particle Size Distribution for SC (average of 4 runs)

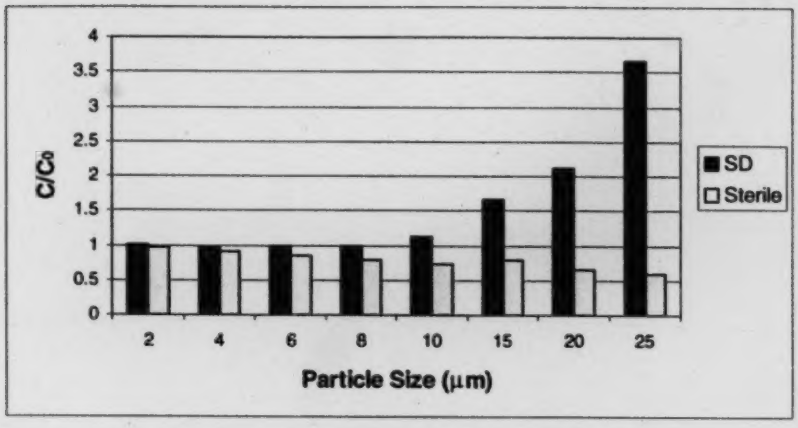

Figure E 20: Change in Particle Size Distribution for SD

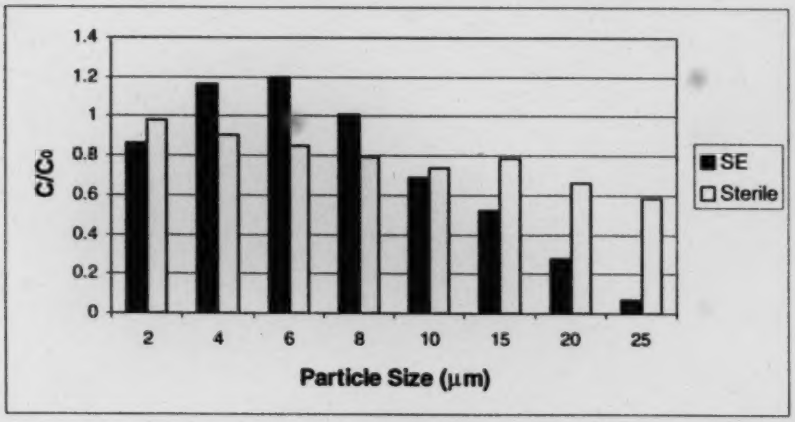

Figure E 21: Change in Particle Size Distribution for SE

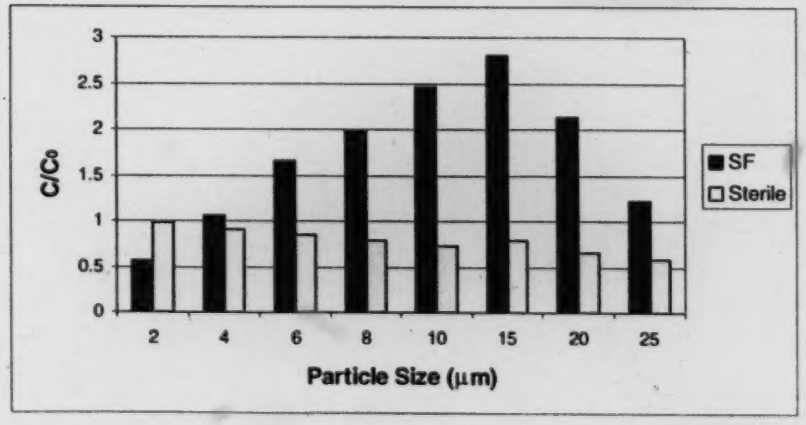

Figure E 22: Change in Particle Size Distribution for SF (average of 4 runs)

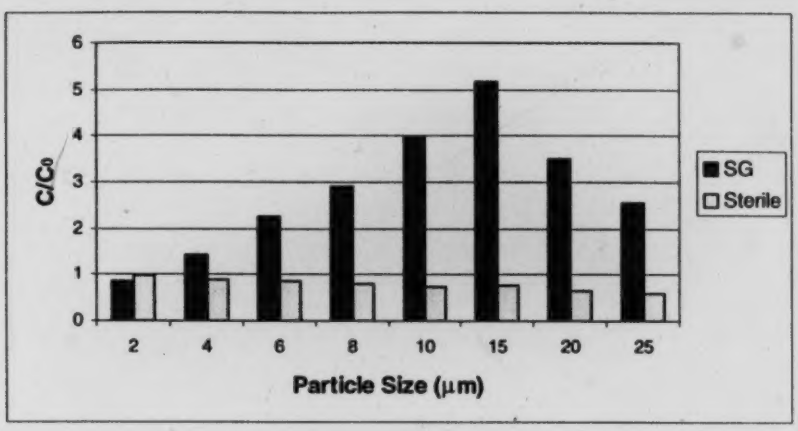

Figure E 23: Change in Particle Size Distribution for SG (average of 4 runs)

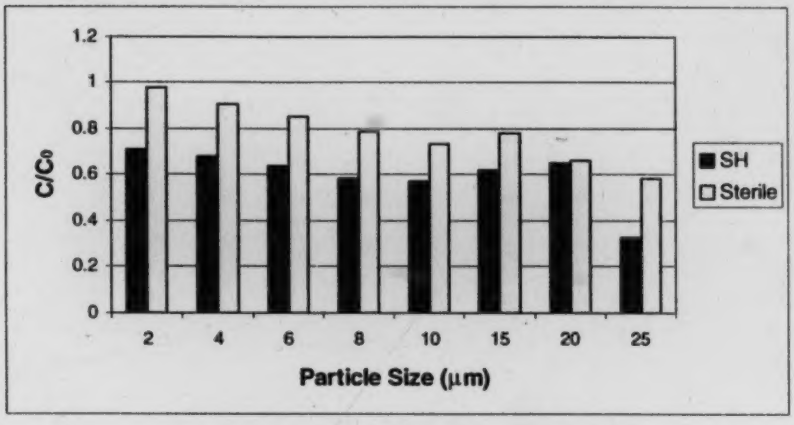

Figure E 24: Change in Particle Size Distribution for SH

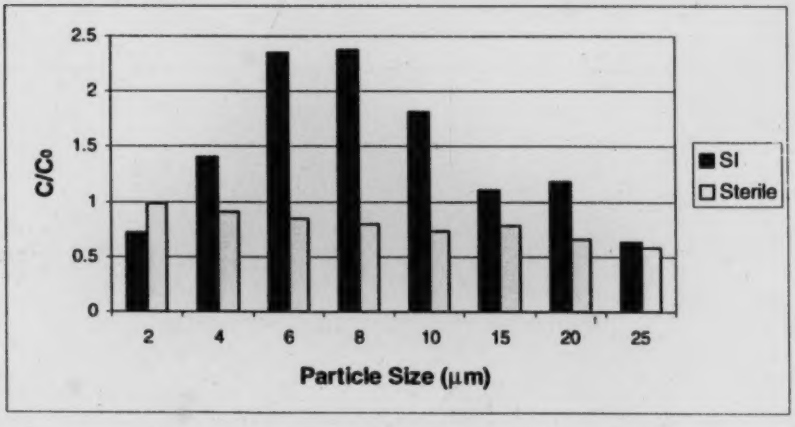

Figure E 25: Change in Particle Size Distribution for SI

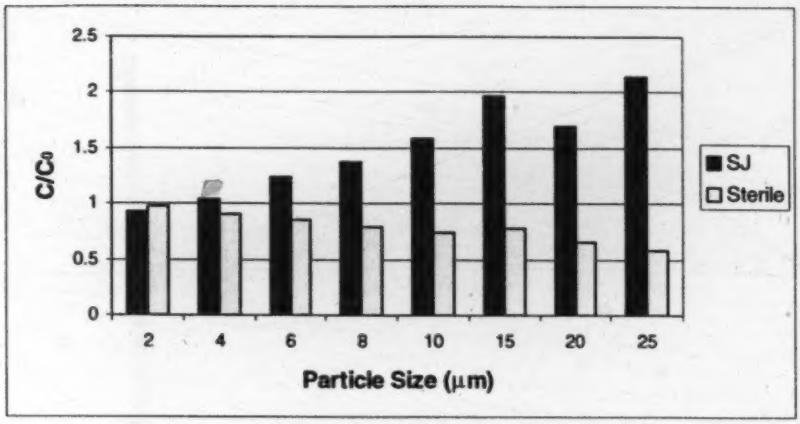

Figure E 26: Change in Particle Size Distribution for SJ

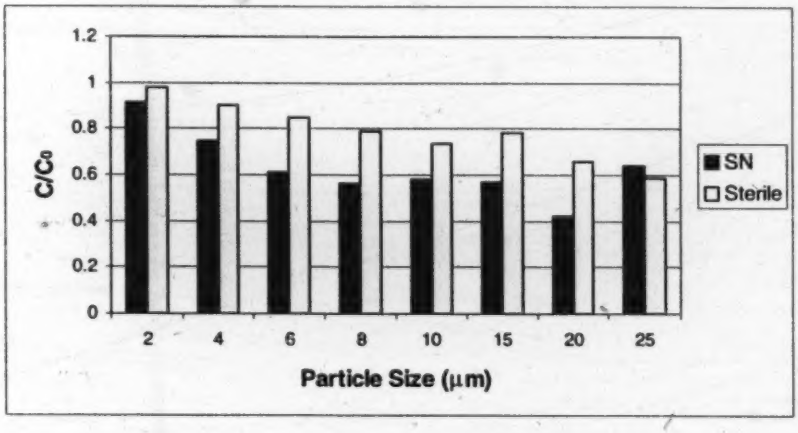

Figure E 27: Change in Particle Size Distribution for SN

APPENDIX F:
Repeated Runs for Chosen Isolates

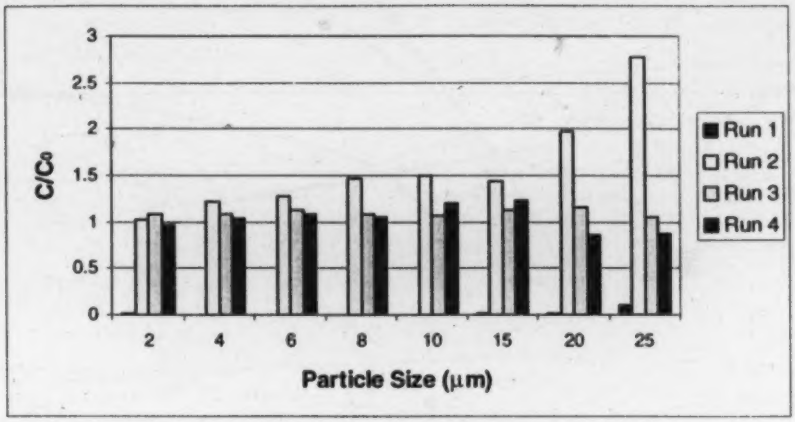

Figure F 1: Individual Runs for HC

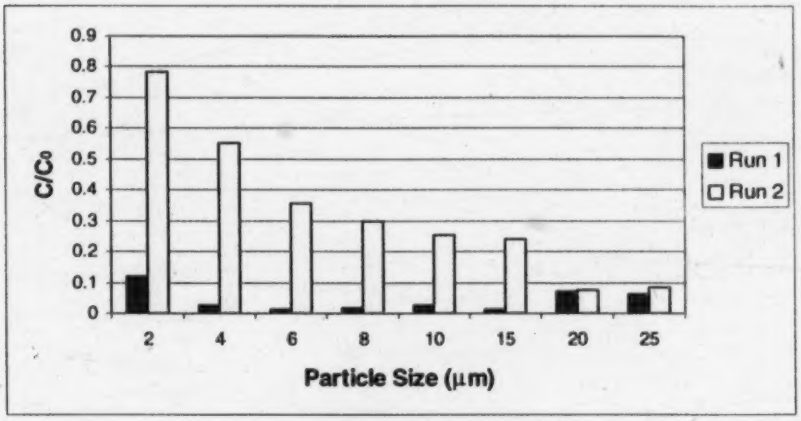

Figure F 2: Individual Runs for HH

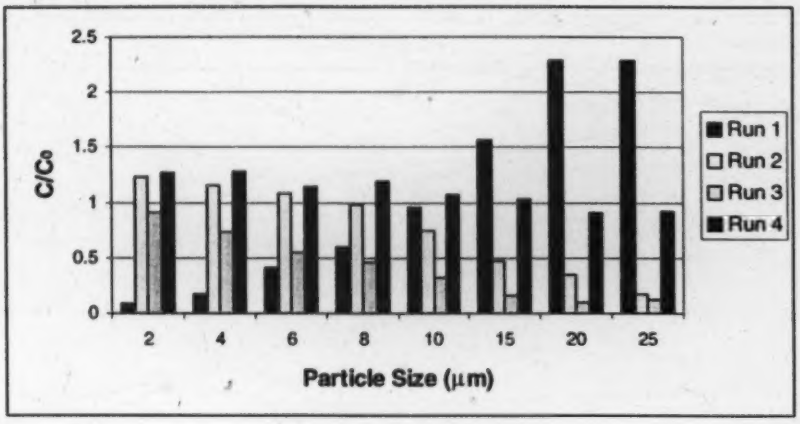

Figure F 3: Individual Runs for HJ

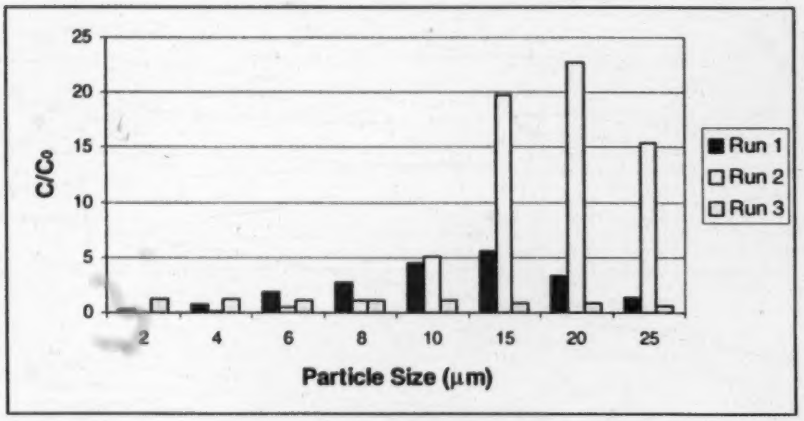

Figure F 4: Individual Runs for HL

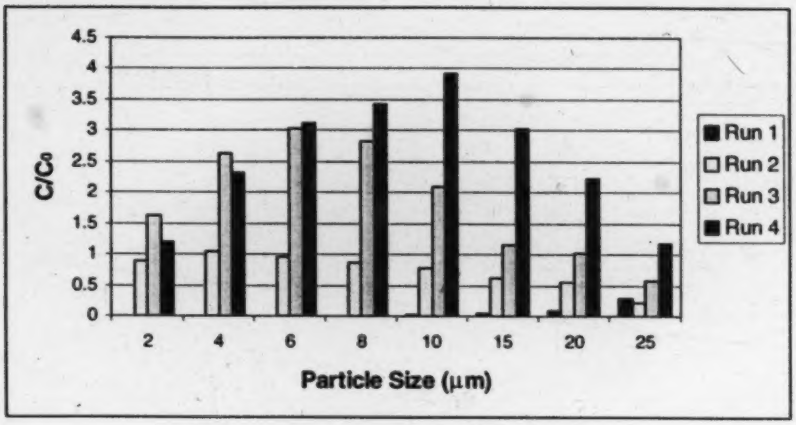

Figure F 5: Individual Runs for SC

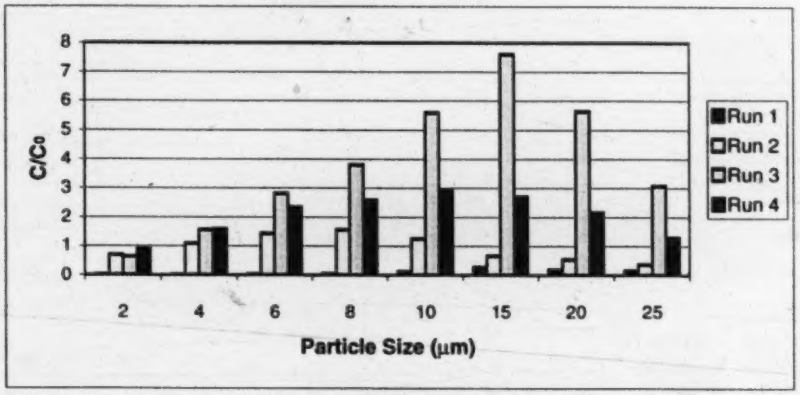

Figure F 6: Individual Runs for SF

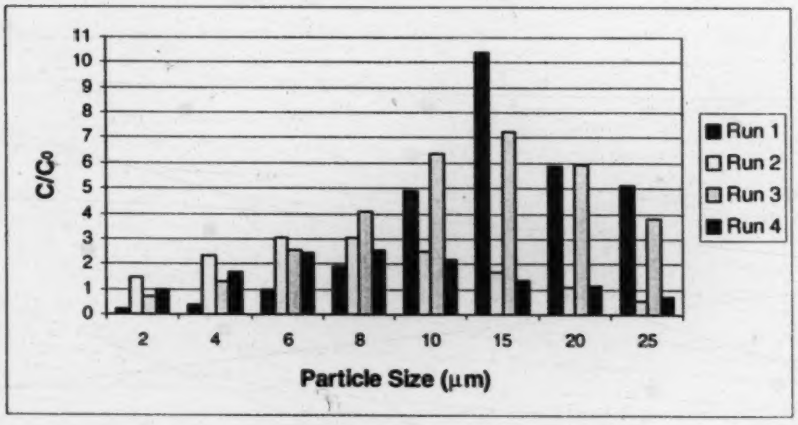

Figure F 7: Individual Runs for SG

APPENDIX G:
Repeated Runs for Controls

Table G 1: Tabulated Values of Four Sterile Runs

Particle Size	C/C_0 (1)	C/C_0 (2)	C/C_0 (3)	C/C_0 (4)	C/C_0 (ave)	std dev
2	1.029	0.899	0.859	1.128	0.979	0.12
4	1.120	0.763	0.674	1.067	0.906	0.22
6	1.237	0.660	0.519	0.987	0.851	0.32
8	1.184	0.626	0.431	0.923	0.791	0.33
10	1.116	0.626	0.374	0.830	0.737	0.31
15	1.029	0.780	0.397	0.931	0.784	0.28
20	0.977	0.548	0.374	0.742	0.660	0.26
25	0.362	0.902	0.415	0.666	0.586	0.25

Table G 2: Tabulated Values of Three Untreated Runs

Particle Size	C/C_0 (1)	C/C_0 (2)	C/C_0 (3)	C/C_0 (ave)	std dev
2	0.827	0.968	0.985	0.926	0.09
4	1.222	0.839	0.953	1.005	0.20
6	1.610	0.720	0.968	1.099	0.46
8	1.870	0.744	1.299	1.304	0.56
10	2.147	1.080	1.786	1.671	0.54
15	2.927	1.489	3.456	2.624	1.02
20	3.575	2.340	3.794	3.236	0.78
25	5.422	2.191	3.708	3.774	1.62

Table G 3: Tabulated Values of Six PME Runs

Particle Size	C/C_0 (1)	C/C_0 (2)	C/C_0 (3)	C/C_0 (4)	C/C_0 (5)	C/C_0 (6)	C/C_0 (ave)	std dev
2	0.401	0.463	0.379	0.142	0.301	0.170	0.309	0.13
4	0.533	0.679	0.549	0.178	0.536	0.264	0.457	0.19
6	0.804	1.237	0.979	0.415	1.153	0.532	0.853	0.33
8	0.951	1.708	1.254	0.662	1.761	0.836	1.195	0.46
10	1.048	2.557	1.451	1.385	3.021	1.807	1.878	0.76
15	0.883	3.366	1.038	3.282	5.058	4.409	3.006	1.72
20	0.647	2.628	0.660	4.025	4.026	5.525	2.918	1.98
25	0.245	1.711	0.328	5.937	2.738	4.357	2.553	2.27